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SYMPOSIUM ON THE PHYSICS, CHEMISTRY, AND BIOLOGY OF THE ATMOSPHERE

BY INVITATION OF THE COMMITTEE ON ARRANGEMENTS FOR THE ANNUAL MEETING

Read before the Academy, April 27, 1959

CONTENTS

CHAIRMAN'S INTRODUCTORY REMARKS . . . . . *Horace R. Byers* 1649
HYDRODYNAMICS OF THE ATMOSPHERE AND NUMERICAL WEATHER PREDICTION—A SYNTHESIS . . . . . *Jule G. Charney* 1650
HEAT EXCHANGE AND WEATHER FORECASTING . . . . *Sverre Petterssen* 1655
ATMOSPHERIC CHEMISTRY AND BROAD GEOPHYSICAL RELATIONSHIPS . . . . . *Bert Bolin* 1663
AIR POLLUTION AND RADIOACTIVITY CIRCULATION PROBLEMS . . . . . *Lester Machta* 1672
PROBLEMS IN BIOCLIMATOLOGY . . . . . *René J. Dubos* 1687

CHAIRMAN'S INTRODUCTORY REMARKS

BY HORACE R. BYERS

THE UNIVERSITY OF CHICAGO

This symposium will serve to show that the atmosphere, in its structure and behavior, poses problems to be approached from a variety of scientific backgrounds.

First, in the paper by Dr. Charney, the atmosphere is treated from the point of view of fluid dynamics. This is the core problem of meteorology—to deduce the flow pattern in terms of the equations of motion for a thermally driven circulation over a rotating spheroid and to predict changes in the flow. Numerical methods have been developed for the modern electronic computers which have taken prediction out of the realm of weather-forecasting lore.

Dr. Petterssen's paper presents the atmospheric motion in terms of the most important constituent carried by the air, namely, water. The character of precipitation over large and small areas, as related to the atmospheric motions, reminds us of the important interactions between the atmosphere and the surface of the earth.

But water is not the only variable substance carried by the atmosphere, as Dr. Bolin's presentation will show. A variety of chemical particles and gases are carried in the circulation, some of which, especially the radioactive ones, serve as

tracers of the flow. The atmospheric involvement in the chemical balance of the earth and especially of the oceans, is an interesting aspect of geochemistry.

We will come a step nearer to considering the effect of the atmosphere on man and man's effect on the atmosphere in Dr. Machta's paper. The atmosphere is a good place to dispose of wastes, but we seem to be carrying a good thing too far. Air pollution and radioactive fallout have become hazards to our health and safety. But radioactive materials in the atmosphere can also serve us in revealing features of air circulation that would otherwise remain hidden.

Man as conditioned by his atmospheric environment has received the attention of our last author, Dr. Dubos. Here we are confronted with evidence that is vital but unclear. A new area of understanding is open to us as biological measurements become more precise and the collection of clinical data becomes more reliable. Man will live to exert more and more control on his environment and to protect himself further from the unfavorable influences of the atmosphere from which he draws his breath.

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*HYDRODYNAMICS OF THE ATMOSPHERE AND NUMERICAL WEATHER PREDICTION—A SYNTHESIS**

By Jule G. Charney

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The principal outlines of a self-consistent theory of the general circulation of the atmosphere have begun to emerge from the work of recent years on the hydrodynamics of the planetary flow patterns in the atmosphere. Observation has shown that the zonal currents are maintained against frictional dissipation by a complex process involving transfer of angular momentum and heat by large-scale eddy motions as well as by axially-symmetrical meridional circulations; theoretical analysis has done much to clarify the mechanism of the formation of the planetary waves and vortices constituting the eddies; and numerical prediction experiments have shown how it is possible to construct simple mathematical analogues of the atmosphere which contain the essential physical elements of the large-scale circulations. While the construction of a comprehensive mathematical theory of these motions remains beyond reach, it is now possible to present a fairly coherent physical picture, or synthesis, of the processes making up the general circulation and to show by mathematical means that this synthesis does in fact explain the gross character of the general circulation.

The reasoning is based upon the following set of principles, which may now be accepted with a certain degree of confidence:

I. The axially-symmetric circulation that would be set up by solar heating on a uniform, rotating, spherical earth is a circular vortex with a slow superimposed meridional circulation carrying heat from equatorial to polar regions. This vortex is stable for axially-symmetric displacements in its gravitational and centrifugal force fields but is unstable for certain types of asymmetric displacements.[1-3] The instability for asymmetric displacements is associated with the decrease of temperature from equator to pole and hence with a supply of potential energy

available for conversion into kinetic energy. It is released when the horizontal scale of a disturbance is so large that, on the average, the particle orbits are less inclined to the vertical than the isentropic surfaces.

II. The asymmetric disturbances which form as a consequence of this instability are in fact the large-scale waves and vortices which act as the important eddy elements in the transfer of angular momentum and heat.[4-6]

III. These eddies are, on the average, stable when regarded as two dimensional perturbations of the zonal flow, i.e., they tend to transfer kinetic energy to the zonal flow via the work of the Reynolds stresses.[7, 8]

IV. The large-scale components of the flow are quasi-geostrophic, except near the equator. Hence one may use the so-called quasi-geostrophic equations to filter out the meteorological "noise" motions in their analysis. These equations provide a simple means to compute the mean zonal flow once the sources of angular momentum and heat are known. The method of computation is based on the principle that the changes in angular momentum and temperature brought about by the real and eddy sources of angular momentum and heat must be such as to maintain a condition of geostrophic and hydrostatic balance. Since these changes are not usually compensating, meridional circulations are required to restore the balance.[9, 10]

V. The gross features of the horizontal distribution of wind and temperature in the atmosphere may be deduced from a mathematical model in which the continuous three-dimensional field of motion is replaced by the distribution in two representative horizontal planes or isobaric surfaces. The coupling between the flow at the two levels is essentially the same as that between two superimposed homogeneous-incompressible layers of differing density. This is the simplest model in which realistic conversions of potential to kinetic energy can take place. It has been used successfully in the investigation of baroclinic instability and in the treatment of the general circulation by numerical integration of the equations of motion.[11, 12]

The underlying problem of the general circulation may be stated in purely hydrodynamical terms as follows: A distributed heat source decreasing in intensity from equator to pole produces a slow, axially-symmetric, convective circulation with a poleward temperature gradient and a zonal wind field increasing in westerly intensity with height. This flow is unstable for small-amplitude wavelike perturbations, which therefore grow to finite amplitude and interact with the zonal velocity and temperature fields. It is required to calculate the mean properties of the final state of motion and also, if possible, the nature of the finite-amplitude perturbations.

One may distinquish two possible classes of motion: (1) The secondary wave disturbances may themselves be stable and approach a constant-amplitude equilibrium state. (2) Several different disturbance modes may be excited by instability of the primary flow, by instability of the secondary disturbances, or by self-interaction of the secondary disturbances, and these modes may appear as a regularly fluctuating state of motion or as a state approaching fully developed turbulence. Although the atmospheric circulation is better described by a fluctuating state of motion, it resembles the equilibrium state in certain important aspects. Both the stationary and fluctuating states have been produced in laboratory and numerical prediction experiments.[13, 14, 15, 16] By confining attention to the steady-amplitude

regime it becomes possible to introduce a simplifying approximation which greatly facilitates the mathematical analysis: only the nonlinear interaction between the disturbance and the mean flow is taken into account; the self-interaction of the disturbance and the subsequent cascade of interactions are ignored. This approximation can be justified if the perturbation amplitudes are not too large.[17]

Within the limitations imposed by the above approximation, one may envisage the process by which the finite-amplitude regime is established in the following way: Imagine a small wave disturbance to be superimposed on the steady symmetric flow produced by the symmetric heating. In the initial stages its interaction

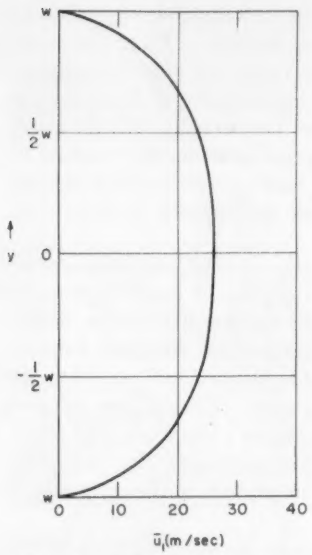

FIG. 1.—The zonal velocity at 250 mb (level 1) in the symmetric convective regime.

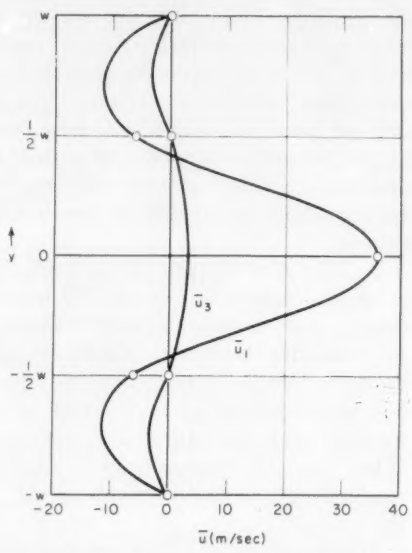

FIG. 2.—The zonal velocities at 750 mb (level 3) and 250 mb (level 1) in the presence of the first unstable asymmetric mode.

with the mean motion may be ignored. But as its amplitude grows, at first exponentially, it begins to distort the main flow, directly through the action of the Reynolds stresses and eddy conduction of heat, and indirectly through the action of the forced meridional circulations. At length the mean flow becomes so modified that the rate at which the perturbation receives energy from the mean flow is just balanced by the combined rates at which it loses potential energy through radiative temperature equalization and kinetic energy through the action of Reynolds and frictional stresses. The form of the disturbance is first calculated from the first-order instability theory; the modification of the mean flow as a function of an undetermined amplitude is next calculated by means of the quasi-geostrophic heat and momentum equations; and finally the amplitude is determined by making use of the energy balance relationships.

The results of calculations are presented for a two-level atmospheric model in which the atmosphere is assumed to be transparent to solar radiation and "grey"

to terrestrial radiation, and the earth is assumed to have zero heat capacity. Energy dissipation by small-scale eddies is provided for in this model by a correspondingly simplified frictional mechanism, and the geometry is simplified by assuming that the flow takes place on a periodic Rossby "β-plane," bounded to the north and south by vertical walls. The zonal velocity profiles $\bar{u}$ are calculated at the 250 mb and 750 mb levels, which are denoted by the subscripts 1 and 3 respectively.

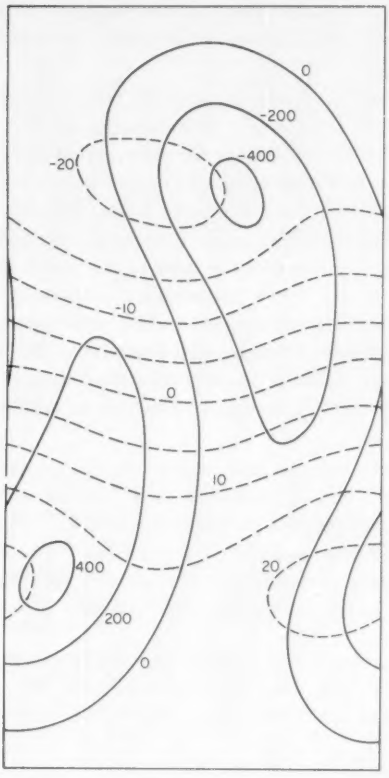

FIG. 3.—Contours of the 750 mb surface labeled in feet (solid lines) and isotherms at 500 mb labeled in degrees C (dashed lines).

FIG. 4.—The zonal velocities at 750 mb (level 3) and 250 mb (level 1) in the pressure of the second unstable asymmetric mode.

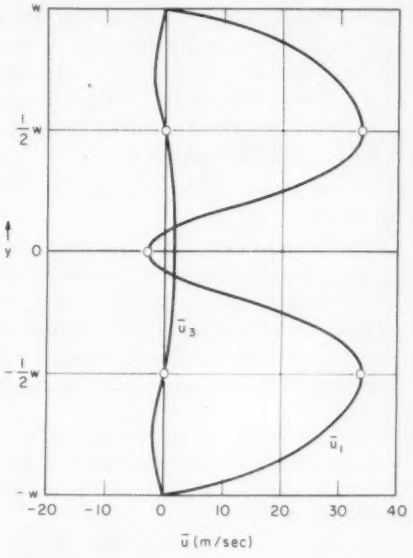

Figure 1 shows the zonal velocity at 250 mb calculated for the steady axially-symmetric vortex produced by the solar heating. It is plotted as a function of the meridional coordinate, y, which varies from the equator (y = −W) to the pole (y = W). As a consequence of the model assumptions, the 750 mb velocity is zero in the symmetric vortex.

When asymmetric eddies are introduced the eddy exchange process drastically modifies the zonal velocity profiles. The modified profiles are shown in Figure 2. In place of a broad band of westerlies at 250 mb there is now a westerly jet in middle latitudes and weaker easterly jets near the walls; and at level 3, where before the zonal velocity was zero, there is now a band of westerlies at middle latitudes and easterlies to the north and south. The corresponding temperature profile is changed

from one in which the temperature decreases uniformly from wall to wall to one in which the variation is concentrated in narrow bands at middle latitudes and near the walls. The eddy processes are very efficient in transferring heat. The total pole-to-equator temperature contrast under conditions of purely radiative equilibrium is 93°. This is reduced to 74°C by the symmetric convective circulation and to 26°C by the eddy processes. The streamlines at the level 750 mb and the temperature at the level 500 mb are shown in Figure 3. The patterns correspond well to an extra-tropical cyclone in the early or middle stages of development and have about the same degree of verisimilitude as is found in the more elaborate numerical machine calculations.

The calculations also indicate the existence of a second unstable perturbation mode having a smaller rate of amplification than the first. If this mode had been selected instead of the first, its interactions with the symmetric flow would have given rise to the zonal velocity profiles shown in Figure 4, which are seen to be very different from those in Figure 2. The differences is due primarily to the fact that the northward eddy flow of heat in the second mode has a maximum at the central latitude. As a consequence there is an eddy convergence of heat to the north of this latitude and a divergence to the south, with a rise in temperature to the north and a fall to the south to allow for radiative compensation. This produces a diminished increase of wind with height at middle latitudes and therefore a diminished zonal wind at upper levels. In contrast, the eddy flow of heat in the first mode is such as to produce an increase in the poleward temperature gradient at middle latitudes.

The actual zonal flow observed in the atmosphere and, under certain circumstances, in laboratory and numerical models can be described as a "vacillation" between two extremes similar to the ones represented by Figures 2 and 4. This suggests that the motion is more suitably described as a shuttling of energy between these modes and the mean flow. The logical extension of the present analysis should therefore include means for dealing with several disturbance modes.

* Summary of the paper given before the National Academy of Sciences; the complete text will be published in the Rossby Memorial Volume (New York: Rockefeller Institute Press, 1959).

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*HEAT EXCHANGE AND WEATHER FORECASTING**

BY SVERRE PETTERSSEN

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Introduction.—Until comparatively recent times it was customary to regard the larger migratory weather systems, the cyclones and anticyclones of middle latitudes, as more or less self contained entities whose movement, growth, and decay were determined almost entirely by the conditions within the system itself. This attitude was largely a consequence of limitations imposed by the horizontal and vertical extent of our network of observing stations. As our observing systems expanded during and after the Second World War, it became increasingly clear that, in many respects, the mobile weather systems are parts of a much larger whole, which often may be the entire hemispheric circulation.

The number of different viewpoints concerning one phenomenon which can be maintained in peaceful coexistence depends upon the amount of ignorance that prevails. At times it has been maintained that differential heating due to heat sources and sinks associated with the topographical features of the earth's surface are all-important. At other times the thinking has gone along the lines advocated by Margules (1903), who produced some arguments in support of the view that the energy of the mobile weather systems could be accounted for by a conversion of potential energy into kinetic energy. In the scheme of Margules, heat sources and sinks play no part, except that it is implied that the potential energy has to be restored somehow. Again, at other times, the development of weather systems has been ascribed to the released latent heat of vaporization. Here, as well as in other fields, one finds that without numerical values very little can be refuted.

A very interesting point of view was advanced about forty years ago by Defant (1921). Defant regarded the mobile cyclones and anticyclones of middle latitudes

as "eddies" in the hemispheric motion which, when integrated over a sufficiently long span of time, achieved the mean state and the mean motion of the atmosphere, or what we commonly call the General Circulation. When regarded in this manner, it is apparent, by analogy with the ordinary turbulent processes, that the mean behavior of the atmosphere is not to be represented by the mean values of its individual properties, and that the migratory weather systems must play an important part in preserving the atmospheric balance of heat and angular momentum. All recent investigations of quality have vindicated this point of view; the overwhelming part of the balance is maintained by the mobile systems.

If we are willing to accept this finding, we should also be willing to revise our approach to research on forecasting and general circulation problems. In earlier years it was thought that a thorough understanding of the behavior of weather systems must wait upon a satisfactory solution of the problem of the General Circulation; it is now apparent that the reverse is the case—a thorough understanding of the General Circulation must wait upon the emergence of a satisfactory understanding of the mobile weather systems, for the former is very little but an integration of the latter.

I believe, therefore, that it is right to say that the time seems past when it would be reasonable to treat the mobile weather systems as manifestations of adiabatic processes, and, at the same time, search for energy sources and sinks to account for the General Circulation.

The Properties of the Earth's Surface.—For reasons which are now well understood, the atmosphere communicates with the sun mainly with the earth's surfaces as a converter of radiation into sensible heat. It is of interest, therefore, to consider the bulk properties of various types of surface substances. In doing so it is well to note that the heat capacities of the air and the surface substances are only of indirect interest. What matters is the *conductive capacity*, or the ability of the substance concerned to conduct the heat away from the interface where it is supplied.

If ρ is the density and c the specific heat, the heat capacity is ρc, and the conductive capacity is $\rho c\sqrt{K}$, where K is the thermal diffusivity; in the case of solids K is the molecular value, and in the case of fluids it is eddy value.

TABLE 1

PHYSICAL PROPERTIES OF TYPICAL SUBSTANCES*
(cgs-cal. units)

Substance	Heat Capacity, ρc	Conductive Capacity, $\rho c\sqrt{K}$	Conductive Capacity, Typical	Surface to Stirred Air Ratio
Snow, new	0.03	0.002	0.002	0.02
Snow, old	0.22	0.012	0.01	0.1
Sand, dry	0.3	0.011		
Sand, wet	0.4	0.04		
Sandy clay (15% water)	0.6	0.037		
Soil, organic	0.57	0.04	0.04	0.4
Soil, wet, marshy	0.7	0.038		
Ice	0.45	0.05		
Water, still	1.0	0.039		
Water, stirred	1.0	0.32–17†	10	100
Air, still	0.0003	0.0001	..	..
Air, stirred	0.0003	0.01–1.0	0.1	..

* Summarized from Priestley (1959).
† Increasing from highly stable to neutral equilibrium.

In Table 1 are shown the thermal properties of some typical surface substances. If we exclude newly fallen fluffy snow and, for the moment, disregard the mobility of the ocean waters, we see that we have, essentially, only two groups of surface substances. The first group contains the snow fields and the fields of dry desert sands, with a conductive capacity of about 0.01. The second group contains all common soils, the ice fields, and the oceans, with a conductive capacity of about 0.04. If the oceans were still, there would be hardly any difference between ocean and continents as far as heat exchange is concerned.

The difference between oceans and continents is entirely due to the mobility along the vertical of the water. Although the conductive capacity of stirred water may vary within a considerable range, we may assign 10 as a typical value of the ocean surface in windy regimes. We see then that the conductive capacity of the oceans is about 100 times larger than that of air. Consequently, we may regard the ocean as an infinite sink of heat—in other words, the air must adapt its temperature to the ocean surface. As far as air and land masses are concerned the difference is not so large (0.1 against 0.04). Here it is more nearly true to say that it is the temperature of the land that yields to the temperature of the air. However, since both vary considerably, it is evident that if heat exchanges are to be incorporated in prediction models, it will be necessary to predict also the manner in which the heat is shared between the atmosphere and the earth. Thus, in order to arrive at a prediction for the atmosphere based upon the equations of physics, we must produce, concurrently, predictions for the ocean and land masses. Forecasting schemes based upon statistical operations could possibly circumvent this complexity.

Computation of Heat Exchanges.—When we deal with the large-scale processes of the atmosphere it is usually satisfactory to discuss the eddy heat exchanges in terms of certain bulk properties, and we need not concern ourselves with such details as are customary in micrometeorology. In fact, we are here concerned not with the microprocesses themselves, but with their integration into large-scale influences.

It has been shown by Priestley (1955) that when the ground is heated under calm but otherwise average conditions, the eddy flux of heat H obeys the condition

$$\frac{H}{\rho c_p (g/\theta)^{1/2} \left|\partial\theta/\partial h\right|^{3/2} h^2} = \text{constant (dimensionless)}.$$

Here,

ρ = density of the air
c_p = specific heat at constant pressure
g = acceleration of gravity
θ = potential temperature
h = height above the ground

We may write this as

$$\overset{*}{H} = \text{constant} \tag{1}$$

where H is dimensionless measure of the eddy flux of heat. The heat flux described by equation (1) results from buoyancy convection, and it is convenient to refer to it as *fully free* convection.

In the presence of some wind there is a considerable measure of dynamic control of the size and structure of the buoyant elements, and this will give rise to additional vertical motions. The control here is by the shear along the vertical of the wind and the relative contributions of shear and buoyancy may be cast into nondimensional form by use of the Richardson number Ri. For this kind of convection we have (Batchelor, 1953; Priestley, 1955)

$$\overset{*}{H} = f(Ri) \tag{2}$$

The heat convection described by equation (2) may be called *fully forced* convection.

It is of interest to note that equation (1) is the limiting case of equation (2) for $-Ri = \infty$; that is, when there is no wind shear, or no wind. In practice it is found that the fully free regime exists also in light winds. However, for such winds as accompany the mobile weather systems, we may safely use the equation of the fully forced regime. In this regime, equation (2) may be written

$$H = AV(T_s - T_a) \tag{3}$$

where $T_s - T_a$ is the difference in temperature between the underlying surface and the air, V is the wind speed, and A is a constant which has been determined from heat balance considerations (Jacobs, 1942) and from aerodynamic theory. A formula similar to (3) holds for evaporation.

Heat Exchanges and Motion Systems.—A number of statistical investigations have shown that the motion systems of the atmosphere are modified—and often substantially so—by heat sources and sinks. However, when we deal with mobile systems we have, in general, no way of determining how much of the kinetic energy is due to heat sources and how much is due to conversion of potential energy into kinetic energy.

A rather unique case, in which the heat-source effect was present in isolation, occurred over the Great Lakes region in February, 1958. In this case a vast mass of very cold arctic air streamed southward and occupied a major part of North America to the east of the Continental Divide. The polar front, with its baroclinic region, was pushed onto the North Atlantic, and there was no indication of conversion of potential energy into kinetic energy over the Great Lakes and surrounding areas.

About 48 hours after the invasion of the arctic air, an area of low pressure with cyclonic circulation began to form over the Great Lakes, where the temperature of the air was 20 to 40°F lower than the water temperature. The general aspects of the synoptic situation are shown in Figure 1. Here, Chart A shows the developing and Chart B shows the fully established low pressure system, while the dashed lines indicate the geostrophic vorticity. In both cases one may imagine a depression of the isobaric surfaces over the Great Lakes superimposed upon a general pressure gradient directed mainly from west to east.

Using a method first developed by Wadsworth and Bryan (1948), the pressure field was resolved into a number of polynomials. By removing the two polynomials which represent a constant gradient across the chart, the residuals shown in the lowest row of charts (Fig. 1) emerged. Furthermore, by using a technique which has been developed by Sangster (1959) and is related to that used by Kelvin (1849) to prove his minimum energy theorem, it is possible to isolate the rotational motion. The result is shown in the middle row of charts.

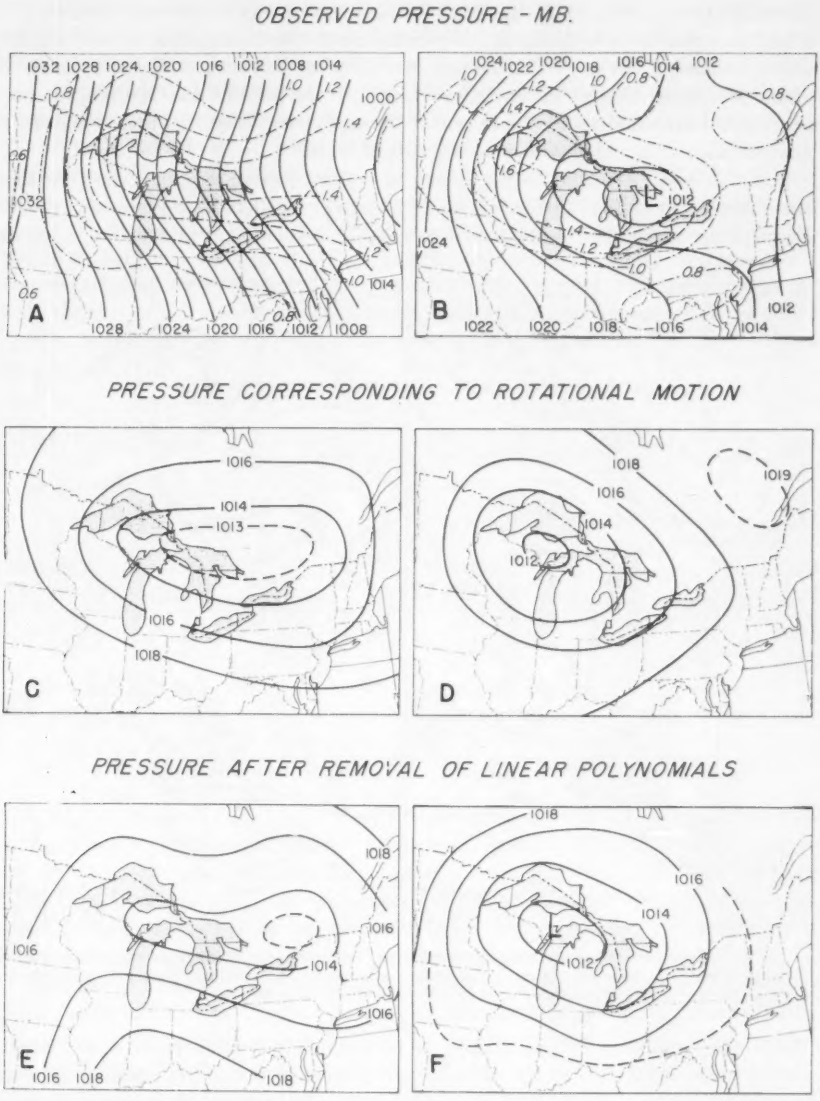


FIG. 1.—Chart A shows the mean pressure and the relative vorticity during the 48-hour period (Feb. 8 to 10, 1958) when a cyclonic system developed over the Great Lakes. Chart B shows the mean pressure distribution and the relative vorticity during the following 72-hour period (Feb. 11 to 14, 1958), when a definite pressure center was present. Charts C and D show isobars for the same periods, representing the rotational motion. Charts E and F show the pressure distributions corresponding to Charts A and B, after removal of the linear polynomials.

There can be little doubt that we have here, for the first time, succeeded in isolating a system of atmospheric motion which is almost exclusively due to a heat source

of topographical nature. In this case the heat source was exceedingly intense, and it had over four days to produce. Nevertheless, the motion system did not acquire much intensity. In a well developed extratropical storm the relative vorticity is about four times that of the earth's surface. In the present case the relative vorticity never exceeded about 80 per cent of the earth's vorticity, and the strongest velocity of the rotational motion was only 8 to 10 m sec^{-1}.

Some evidence collected from analyses of mobile systems supports the view that the direct effect of heat sources is not very large as far as motion is concerned. In Figure 2 are shown two successive stages in the development of a major storm on

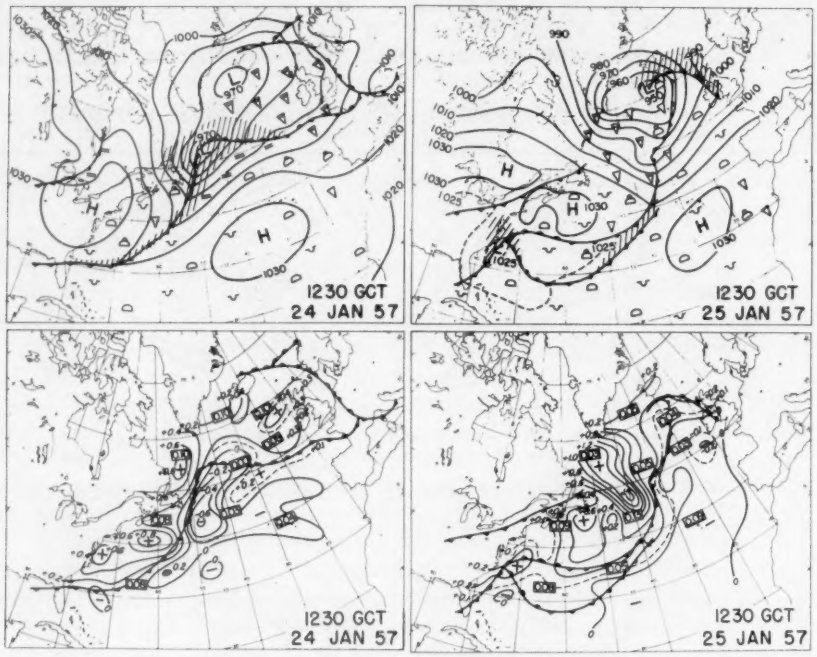


FIG. 2.—Upper charts show two successive stages in the development of a major storm on the Atlantic Ocean. Lower charts show the eddy exchange of heat (cal cm^{-2} min^{-1}) between the oceans and the air. Figures in squares are the radiative exchanges.

the North Atlantic. It is of interest to note that the heating patterns are largely out of phase with the motion patterns, the largest heat transfers occurring in the rear of the storms. As a result, we find that, on the average, the rear of a storm is more intense than its front section, but the difference is not strikingly large.

Figure 2 shows another interesting feature. In the lower row of charts the numbers in squares indicate the radiative flux of heat from the ocean to the atmosphere. It will be seen that the amounts are about one order of magnitude smaller than the eddy fluxes. Moreover, the patterns of the eddy fluxes are tuned to the motion system, while the radiative fluxes show no pattern at all—except that the values decrease northward. The examples shown here are typical, and we may conclude

that, as far as forecasting for relatively short intervals of time (say, one or two days) is concerned, we may ignore the radiative fluxes but not the eddy fluxes. Over extended periods (say, one or two weeks) the radiative fluxes are important, for they tend to maintain a northward gradient of the available potential energy of the atmosphere, and to re-establish such a gradient each time stored potential energy becomes converted into the kinetic energy of mobile storms.

Heat Exchange and Weather Systems.—While it is true that the direct effect of heat sources on the development of motion systems is not spectacular, the same is not true of weather systems; here we find influences which are sometimes overwhelming. This is true of the development of fog, showers, and thunderstorms, and it is possible that nonadiabatic effects contribute through a wide range of weather phenomena. As in the case of motion systems it has been difficult to study the nonadiabatic effect alone. However, in a few cases it has been possible to isolate the effect of heat sources.

TABLE 2

PERCENTAGE PROBABILITY OF WEATHER TYPES IN INTERVALS OF EDDY FLUXES OF HEAT (NORTH ATLANTIC OCEAN JANUARY 18–25, 1957)

Heat Flux Cal cm^{-2} min^{-1}	Weather Systems				Number of Cases
	HCA	MCA	LCA	F&S	
Under −0.2	0	0	0	100	9
−0.2 to 0.0	0	11	63	26	168
0.0 to 0.2	3	31	63	3	510
0.2 to 0.4	15	47	38	0	154
0.4 to 0.6	42	36	22	0	79
0.6 to 0.8	48	36	16	0	53
0.8 to 1.0	78	17	5	0	21
over 1.0	100	0	0	0	17
Number of Cases	139	280	522	70	1011

The weather systems are indicated by the following symbols:
HCA—heavy convective activity; heavy showers, squalls, hail, thunderstorms, etc.
MCA—moderate convective activity, cumulonimbus with scattered showers.
LCA—light convective activity, mainly cumulus humilis and stratocumulus, no showers.
F&S—overcast with low stratus and fog.

Table 2 shows some data for the North Atlantic Ocean. It will be seen that when the heat flux from the air to the water numerically exceeds 0.2 cal cm^{-2} min^{-1}, fog or low stratus reigns supreme, regardless of other influences. Similarly, when the heat flux is from the ocean to the atmosphere and exceeds 1.0 cal cm^{-2} min^{-1}, heavy convective activity (squalls, thunder, and hail) is the only possibility. Between these extremes we find a wide dispersion, reflecting complex influences.

The heat source effect on clouds and weather is perhaps most clearly indicated along shores and coasts. Figure 3 shows the amounts of precipitation that fell in connection with the cases shown in Figure 1. In many places the precipitation maxima are determined by the slopes of hills and mountains, but in northern Indiana and southwestern Michigan the country is flat. Nevertheless, distinct maxima of precipitation are found about 40 km from the shore, and these maxima are about as large as the orographic ones. This displacement can be accounted for (see Pettersen and Calabrese, 1959) by considering the change in frictional drag and acceleration that the air undergoes as it passes from a neutral equilibrium over the warm water to a highly stable stratification over the much colder land. About 24 hours after the time to which Figure 3 refers, the winds changed to a northerly direction so that the trajectory of the air over open water increased appreciably. A snow

depth of fifty inches accumulated within 24 hours (Fig. 4), and all of it can be ascribed to the heat and water vapor which poured forth from Lake Michigan into the cold air.

Concluding Remarks.—The most obvious conclusion that can be stated at the present time is that our knowledge of the heat sources that are available to the mobile motion and weather system is very meager, and so is our knowledge of how even known sources would affect such systems. Second, as far as motion systems are concerned, it would serve no useful purpose to include in our prediction schemes the effects of heat sources and sinks unless we could also include the effects of friction, for with heat source and no frictional dissipation, the atmosphere would soon misbehave. On the other hand, the inclusion of the effects of heat sources and sinks in predictions of the behavior of weather systems would seem to yield rewarding results.

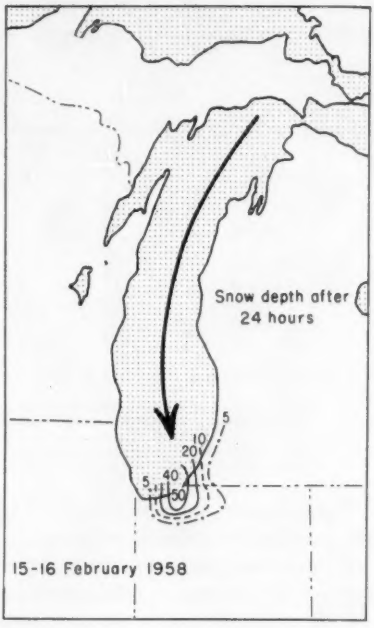
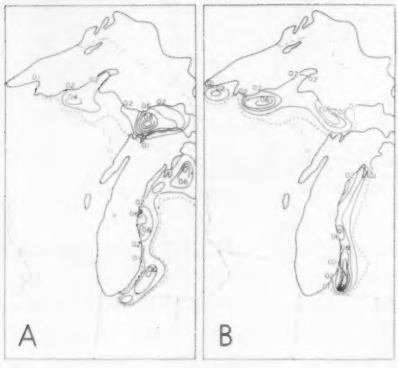


FIG. 4.—Snow depth (in inches) after 24-hour period of exceptionally heavy lake flurries. (Courtesy George C. Williams, U. S. Weather Bureau, Chicago District Center.)

FIG. 3.—The total amounts of precipitation during the periods to which Charts A and B in Fig. 1 refer. Note the maxima of precipitation over the flat land in northern Indiana and southwestern Michigan.



Though the problems are numerous and intricate, it is not difficult to identify the broad sectors within which improvement may be sought. First, I think further studies of the type I have outlined here will provide much of the factual knowledge from which theory will spring. Second, the powerful tool afforded by modern computers should enable us to conduct a variety of numerical experiments with controlled variability, along the lines initiated by Phillips (1956). Third, I am sure we should lose no time in providing, as part of the routine synoptic information, observations on soil and sea-surface temperatures, net radiation, and such other parameters as are needed to attack the problem on a broad basis.

* The research reported in this article has been supported by the U. S. Air Force, under contract no. AF 19(604)-2179, and by the National Science Foundation.

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ATMOSPHERIC CHEMISTRY AND BROAD GEOPHYSICAL RELATIONSHIPS

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Atmospheric chemistry is the science dealing with studies of the composition of the atmosphere, of how the interplay between the atmosphere, the surface of the continents and the oceans, biological activities, man, and last but not least the ever moving atmosphere itself constantly changes this composition as well as the geographical distribution of the various compounds in the atmosphere. It is atmospheric pollution in a very broad sense. We wish to understand the global circulation of these various elements and are not merely interested in the local manifestations as is usually the case when studying industrial pollution. As we shall see, the increasing activities of man are now becoming important factors also on the global scale. Man is interfering more and more with nature. We need to know more about where and how this occurs.

The following *summary* is an attempt to synthesize a number of various observations made in the field of atmospheric chemistry during recent years. It is largely the result of cooperative efforts at the International Meteorological Institute in Stockholm.

Deposition of Atmospheric Pollutants.—Large amounts of sea salt are found in precipitation and in the air everywhere on the earth, but in very different amounts.

Thus Figure 1 shows the amounts of one of the most common constituents in the atmosphere: chloride, as found in precipitation over Scandinavia. A pronounced gradient exists from the sea inland and the prevailing westerlies obviously carry

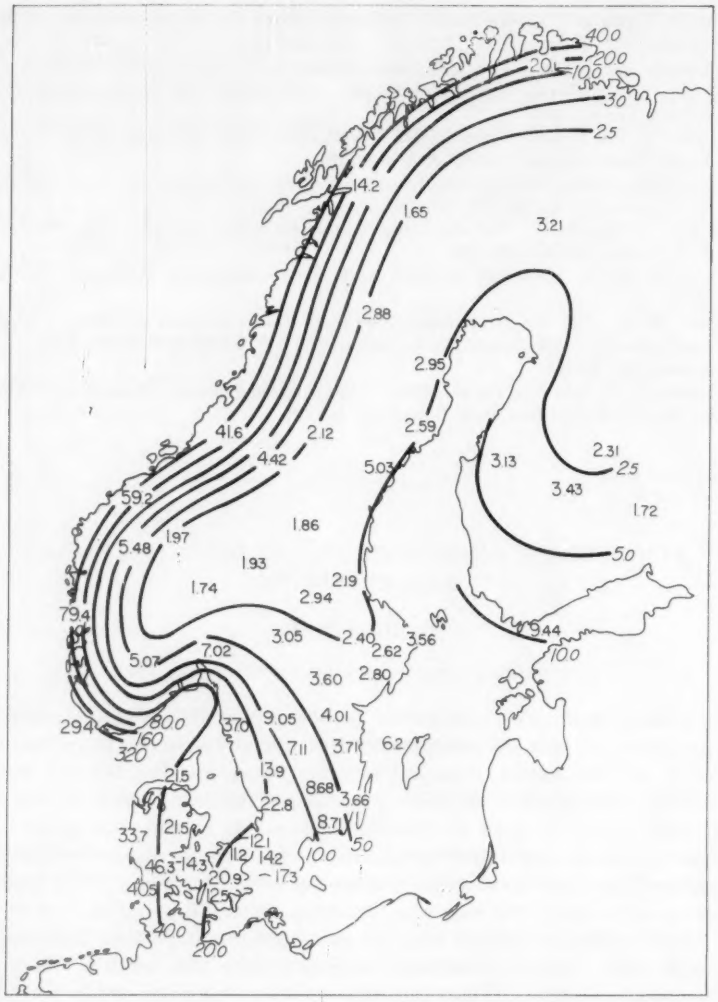

FIG. 1.—Chloride in precipitation in Scandinavia in kg ha $^{-1}$ year $^{-1}$ (from Eriksson, 1959). Average for the years 1955–57.

large amounts of salt in over the continent. In some areas of the earth the salt accumulates and salt deserts result, but in most parts of the world a circulation between the sea and land takes place in that the rivers carry salt back to the sea.

Studying more carefully the amounts of salt transported by the rivers (Fig. 2) one finds that it considerably exceeds the amounts brought to the surface of the earth by precipitation (Eriksson, 1955; 1959). A careful investigation of the possible reasons for this difference excludes the possibility of chemical denudation

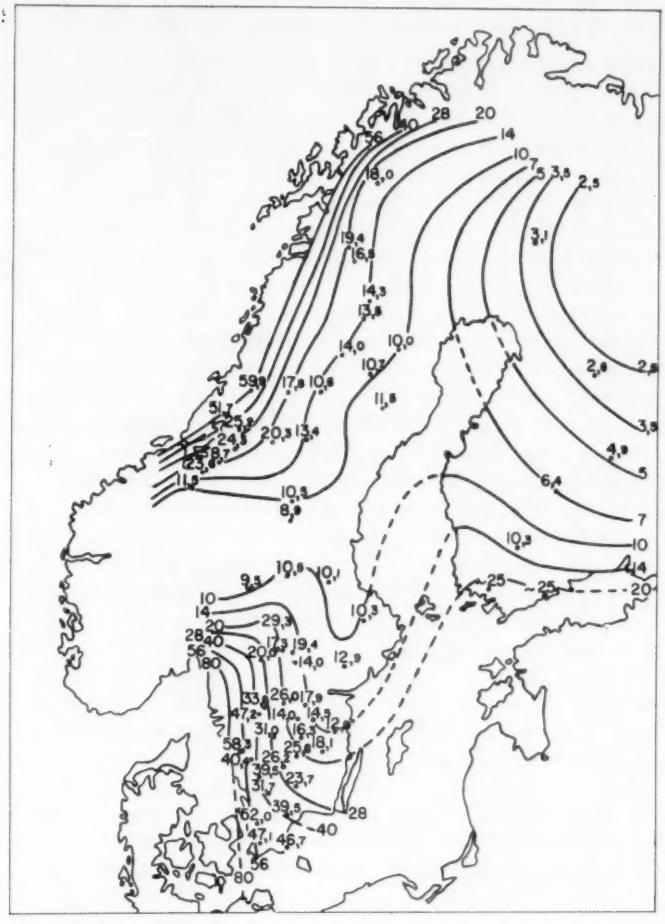

FIG. 2.—Discharge of chloride by Scandinavian rivers in kg ha^{-1} $year^{-1}$ (from Eriksson, 1955).

of the soil. The most probable explanation is instead that much more salt is transferred to the surface of the earth than what is found in the precipitation. The salt is present in the atmosphere in the form of small particles and droplets. In the lowest part of the atmosphere these particles are steadily removed by impaction on vegetation. Particularly the needles and the leaves of the trees

ought to be effective in this respect which is also supported by direct observations. By the removal at the earth's surface in this way a vertical gradient is established in this part of the atmosphere over land (Eriksson, 1959) and a turbulent transfer from aloft becomes possible. The effectiveness of this process can be expressed in a deposition velocity, which is defined as the ratio of the amount deposited over the unit area and in unit time to the amount present in the unit volume of air at a few metres above the surface. Also the *air* concentration of

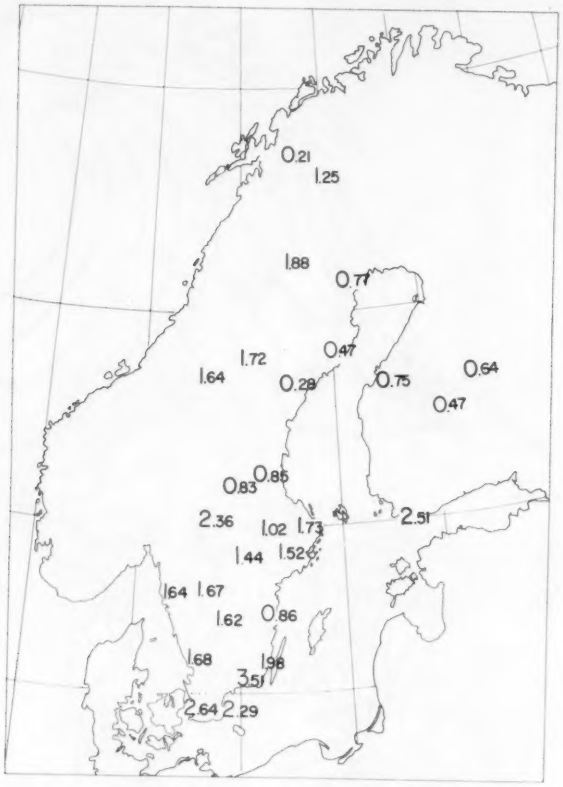

FIG. 3.—Deposition velocity for chloride in units of cm sec.$^{-1}$ (from Eriksson, 1959).

chloride has been measured over Scandinavia and if we make the assumption that the difference between the amounts of chloride discharged by the rivers and the amount deposited by precipitation is transferred to the surface of the earth by such a turbulent process we can compute the deposition velocity in the case of chloride. The result is displayed in Figure 3. One finds an average deposition velocity of 1–2 cm sec^{-1}. Since presumably no chloride is released from the soil it is clear that the net transfer velocity just computed is equal to the gross transfer velocity. This is not always the case for other atmospheric constituents. A

similar computation using the observations of sulphur yields considerably smaller values, in some areas even negative transfer velocities are obtained indicating that a net transfer of sulphur from the soil to the atmosphere takes place. Similar computations for other atmospheric constituents should prove valuable both from the point of view of atmospheric transfer problems and for a better understanding of the different behaviour of various atmospheric constituents.

It is of particular interest to compare the magnitude of the transfer or deposition velocity thus found for chloride with the value obtained from measurements of the deposition of radioiodine, I^{131}. Chamberlain and Chadwick (1953) carried out experiments over an open grass field. I^{131} was released at a certain point at some metres elevation over the ground and the content of the air and the amounts deposited on the grass were measured down wind from the point of release. The deposition velocity computed from these measurements is about 2.5 cm sec^{-1}. Regular measurements of the I^{131} content of the air and grass were also made in England after the Windscale accident in October, 1957 (Dunster, Howells, Templeton, 1959). The values obtained in this case were, however, considerably smaller. The reason for this fact is not quite clear (Bolin, 1959 a). It might be due to less effective vertical exchange in the atmosphere at this time of the year or the different character of the vegetation cover in the summer and the fall. Finally, we do not know the form in which the iodine is deposited and chemical reactions may have occurred in the course of a day or two that affected the deposition velocity.

A third piece of evidence with regard to such transfer processes is obtained from observations of tritium. This is particularly interesting in view of the fact that tritium in the atmosphere is present in the form of water, HTO, and is thus in the gaseous phase, opposite to what is the case for chloride. The series of hydrogen bomb explosions that took place in March, 1954, brought large amounts of tritium into the atmosphere. During the following six to twelve months a marked increase of the amount of tritium in natural waters was observed. In an analysis of these data (Bolin, 1959 b) it was found that the direct molecular exchange of tritium or HTO between the atmosphere and a water surface is very important in studying the tritium budget of a lake. A fairly accurate estimate of the deposition of tritium in some lakes can be made from existing measurements. Let us for simplicity consider Crater Lake, where practically all tritium is transferred by molecular exchange at the water surface and which is comparatively small so that the tritium content of the air is not too much influenced by the presence of the lake. The depth of the lake is, furthermore, so great that the *specific* activity remained low compared with the activity in the air. The transfer back to the atmosphere can therefore be neglected. We get an approximate (probably somewhat too large) value of the tritium concentration of the air by putting it equal to that of the precipitation with due regard to fractionation in the molecular exchange between the water vapour in the air and the droplets. Using the values given by Begemann and Libby (1957) and the assumptions listed above and also previously discussed by the author (Bolin, 1959 b), we find a deposition velocity of about 6 cm sec^{-1}. The uncertainty of this value is obviously quite great and more complete measurements would be of interest in this connection. In view of the differences in the physical process of exchange between the atmosphere and the underlaying surface in the three cases mentioned here it seems likely that *the limiting factor for the trans-*

fer in these cases is rather the turbulent transfer within the atmosphere than the character of the exchange process at the interphase.

Quite a different picture is, on the other hand, obtained if studying carbon dioxide. According to estimates by Craig (1957) and Bolin and Eriksson (1959) the residence time for carbon dioxide in the atmosphere is 3–5 years. Evidently residence time refers to the gross transfer from the atmosphere to the sea, as is also clear from the way it is estimated using radiocarbon. With the knowledge that the homogeneous atmosphere is about 8,000 m we obtain the upper limit of the transfer velocity for carbon dioxide 0.007 cm sec^{-1}. This very small value indicates that the bottleneck for the transfer is either at the interphase between the two media or in the ocean. Further explorations along this line of thinking might be fruitful.

Atmospheric Chemistry as Part of Geochemistry.—Through the ages the atmosphere has played a very active part in the weathering processes occurring on land and this is also the case now. By studying the processes occurring today we can learn something about developments that have taken place far back in time and, vice versa, studies of geological formations on the earth, the composition of the oceans, and the ocean sediments can guide us in our attempts to form a clearer picture of the chemistry of the atmosphere to-day.

Eriksson (1959) has again studied the exchange of matter between land and sea in a way similar to the classical studies by Goldschmidt and others found in the geochemical literature. The chemical composition of igneous rock is quite well known. On the basis of the composition of sedimentary rocks and the amounts of Ca, Na, and K present one can estimate the total amount of igneous rock weathered to about 160 kg/cm^2 of the earth. It is easy to show that Cl and S (as well as B, C, Br, and I) cannot be accounted for by weathering. These so-called "excess volatiles" may have been introduced by volcanic activities or possibly to some extent been present at an early stage of the development of our planet.

Through studies of the composition of river waters we can obtain a fairly good idea of the present rate of weathering, but here a correction for the cyclic salts is necessary, that is, the salts that daily are transported from the ocean in over land and that are brought back by the rivers. Using the observed values of Cl or Na in the river waters to correct for the other constituents of the sea that are transported in a similar way in proportion to their abundance in the *sea*, we find that we still cannot account for the sulphur present in the rivers. Release of sulphur to the atmosphere due to human activities is by no means negligible even on a hemispheric scale but still cannot account for the comparably large amounts found in the river waters. We are indirectly led to the conclusion that sulphur escapes from the ocean in a different way than chloride and sodium. Still the concentrations necessary in the sea water to make this possible are undetectable with present methods of analysis.

The discussion above is only qualitative and the reader is referred to the paper by Eriksson (l.c.) for details. The brief discussion above illustrates, however, how a combination of geochemical and hydrological data can give important clues to problems in atmospheric chemistry. Presumably this can be done in many other ways than indicated here.

Changes of the Carbon Dioxide Content of the Atmosphere.—It was mentioned

above that appreciable amounts of sulphur are brought into the atmosphere due to human interference. Burning of fossil fuel is an even more striking example of human activities that are of a definite geochemical interest. In a few centuries we release into the atmosphere carbon deposited on land over many million years. It is of particular interest to find out whether this excess of CO_2 stays in the atmosphere or is transferred into an increasing vegetation or into the sea. It is well known that the carbon dioxide is of importance for the radiation balance of the earth.

Some twenty years ago Callendar (1938) could show that most likely a noticeable increase of the CO_2 in the atmosphere had occurred. Recent studies by Callendar (1958) and Bray (1959) indicate that this increase in 1955–1956 amounted to about 10 per cent as compared with an output due to combustion of about 14 per cent since the middle of the last century. Studies of the C^{14} distribution in the atmosphere, biosphere, and the sea reveal, however, that the residence time for carbon dioxide in the atmosphere is between 3 and 5 years (Craig, 1957; Bolin and Eriksson, 1959). It has been concluded (Revelle and Süess, 1957) that most of the CO_2 due to combustion has been transferred to the ocean and that a net increase of only a few per cent has actually occurred. It is, however, necessary to consider the dissociation of H_2CO_3 in the ocean to interpret the residence time of five years in the atmosphere with regard to the size of the *net* changes in the reservoirs: the atmosphere and the ocean. The carbon dioxide exists in the ocean in the form of H_2CO_3 or dissolved CO_2, which cannot be distinguished from each other, as HCO_3^{-1} and CO_3^{-2} ions. The partial pressure is proportional to the amount of H_2CO_3 and CO_2 only. Addition of CO_2 to the sea changes the pH and thereby also the degree of dissociation. The percentage increase of the partial pressure becomes about 12.5 times the percentage increase of the *total content* of CO_2 in the water. In view of the fairly slow circulation of the ocean it is then easily understandable that a major portion of the CO_2 released by combustion may still stay in the atmosphere in an approximate balance with the sea in spite of a rapid exchange between the atmosphere and the sea. Let us consider the sea as consisting of two well-mixed reservoirs on top of each other. The upper reservoir, in direct contact with the atmosphere, is the part of the ocean located above the thermocline and constitutes about $^1/_{50}$ of the total sea. This part of the ocean is well mixed due to wind action and convection. The remaining part of the ocean, the deep sea, is also taken as a well-mixed water body in direct exchange with the mixed layer above (Craig, 1957). In a first attempt to study the interplay between the atmosphere and the sea we also neglect the effect of living matter on land. Figure 4 shows the percentage change of carbon dioxide in the atmosphere in 1954 for different values of the exchange rates between the atmosphere and the mixed layer and between the deep sea and the mixed layer. (τ_{a-m} is the residence time for carbon dioxide in the atmosphere with regard to transfer to the sea; τ_{d-m} is the residence time in the deep sea with regard to transfer to the upper layer of the sea; for details, reference is made to Bolin and Eriksson, 1959.) The figure shows that the net increase in the atmosphere is always independent of the precise rate of exchange between the atmosphere and the sea. We also see that the reasonable value of $\tau_{d-m} = 500$ years gives a 10 per cent increase in the atmosphere in good agreement with the estimates based on observations as given by Callendar (l.c.). The results are also quite insensitive for the ex-

act division of the ocean into two such reservoirs as has been assumed. It should finally be added that the transfer of CO_2 from the atmosphere to the ocean of the amount given here should correspond to a decrease of the pH of the surface waters of the sea by about 0.04 units since the middle of the last century, which is hardly detectable in view of the great geographical variations that exist. Further changes in the future may, however, be detectable.

How are these results compatible with the comparably small change of C^{14} in the atmosphere as has been discussed by, for example, Revelle and Süess (1957)?

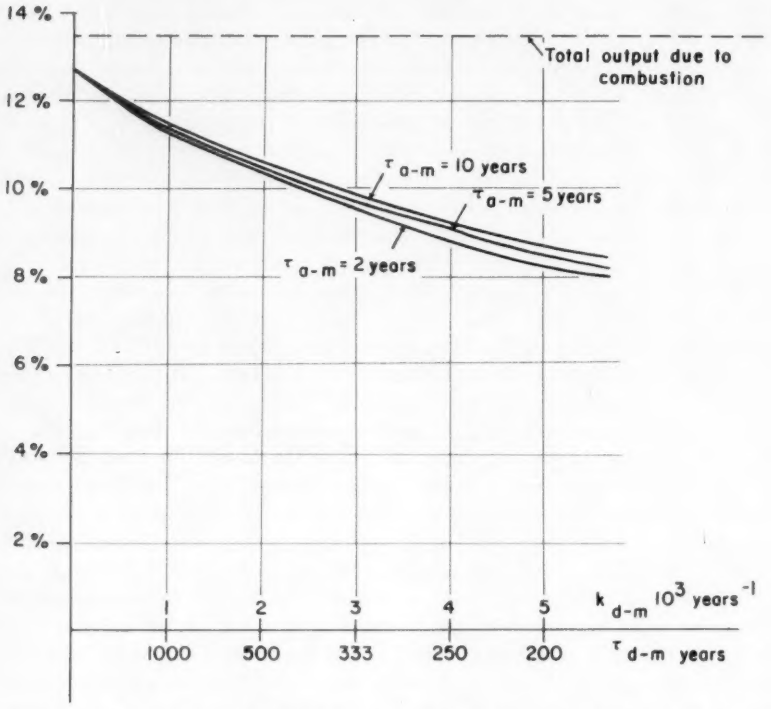

FIG. 4.—Net increase of CO_2 in the atmosphere in 1954 due to release of fossil CO_2 as dependant on the rate of exchange between the atmosphere and the sea and within the sea (from Bolin and Eriksson, 1959).

The fossil carbon is free from C^{14} and one would therefore offhand expect a 10 per cent decrease of the C^{14}/C^{12} ratio in the atmosphere. The change of the dissociation equilibrium in sea water resulting from a transfer of CO_2 to the sea, however, also affects the C^{14} distribution in the two oceanic reservoirs and in the atmosphere. Obviously the C^{14}/C^{12} ratio would be considerably larger in the sea than in the atmosphere if a sizeable increase of CO_2 in the atmosphere has occurred which then would result in a net transfer of C^{14} from the sea to the atmosphere. The rate of this transfer will again be dependent on the exchange times between the various reservoirs. Figure 5 shows the percentage change of C^{14} in the atmosphere and

Figure 6 gives the changes of the C^{14}/C^{12} ratio, the "Süess effect." *It is clear from the last figure that a 7–10 per cent increase of the carbon dioxide in the atmosphere due to fossil fuel combustion is compatible with a 3–4 per cent decrease of the* C^{14}/C^{12}

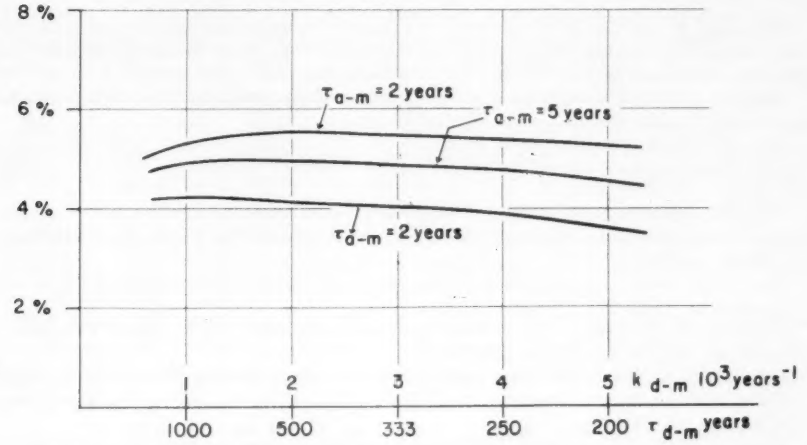

FIG. 5.—The increase of C^{14} in the atmosphere in 1954 by a net transfer from the sea resulting from fossil fuel combustion and the associated change of the dissociation equilibrium in the sea (from Bolin and Eriksson, 1959).

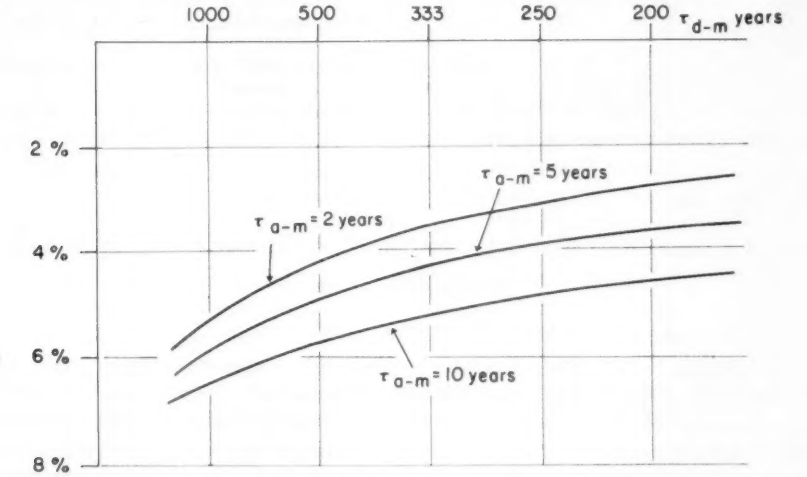

FIG. 6.—The expected value of the Süess effect in 1954 for various rates of exchange between the atmosphere and the sea and within the sea (from Bolin and Eriksson, 1959).

ratio. A more detailed discussion of these results is given in the paper by Bolin and Eriksson (1959) referred to earlier.

It should finally be pointed out that the result outlined above implies a considerable increase of the carbon dioxide content of the atmosphere during the next few

decades with a constantly increasing fossil fuel combustion. A 10 per cent increase by 1954 corresponds to about 40 per cent in the year 2000. It should be possible within a few years to measure directly whether the rate of the increase is that rapid or not.

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AIR POLLUTION AND RADIOACTIVITY CIRCULATION PROBLEMS

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Introduction.—The practicing weather forecaster cares very little about the composition of the air aside from its water vapor content. But, we are now beginning to realize that there is more to meteorology than advising the public whether or not it will rain tomorrow. We live in the atmosphere and it is important that our environment be kept as clean as possible. Man's wastes are now so enormous that in certain areas like Los Angeles the atmosphere is, at times, incapable of providing adequate location dilution. This local city pollution is our immediate concern. But, we do not destroy the wastes by disposing of them downwind of the city. Under some circumstances, the pollution of many cities re-enforce one another and affect areas far removed from the sources. Ultimately, if the exponential growth of industry with its waste disposal problem continues, contamination may become

global and further dilution impossible. If enough pollution is added to potentially affect the whole earth, we must make sure that the atmosphere's cleansing powers will be adequate to keep it habitable.

The consequences of air pollution reach beyond health and agriculture. They affect the weather as well. There is strong evidence that increased atmospheric contamination reduces visibility, modifies electrical conductivity, alters precipitation, and changes the radiation balance. What more subtle and far-reaching effects will result from these and other phenomena are now unknown.

Radioactive pollution is receiving its share of attention today, but rather than describe its hazards, this article will present the benefits which the meteorologist is gaining by using radioactivity as a tracer. There is also great promise in using many of the stable substances in the atmosphere as tracers. Thus, changes of state of water in the hydrologic cycle are reflected in the isotopic ratios of oxygen 16 to 18 and deuterium to protium. For example, evaporation from lakes has been found this way. Many other examples could be given.

This paper will deal with two subjects. First, a review of large scale air pollution problems in meteorology and second, some examples of the ways in which man-made and natural radioactivity is helping the meteorologist to understand the atmosphere.

Air Pollution.—Our present aims in studying the meteorology of air pollution are measurement, understanding, and prediction. Unfortunately, none of these reduces the amount of pollutants put into the atmosphere. But through the proper selection of industrial sites, local contamination can be minimized; through meteorological detective work, an offending culprit can be uncovered; and through forecasting, pollutants can be withheld pending a return to improved dilution conditions. The major effort in meterological research of air pollution is on a micro-scale—city-wide contamination. At present, a meteorological analysis is considered successful if a way is found for the pollutants to be vented into the atmosphere without local difficulties. But is this the solution? By the time the effluent reaches the next city, the diffusion is believed to reduce the concentrations to a satisfactory level. But there are exceptions even today. Dr. Keeling[1] of Scripps watches the carbon dioxide abundance rise from 310 to 340 parts per million as a brown cloud descends on him from the north. Yet, La Jolla is almost 100 miles south of Los Angeles. It is estimated that 15 per cent of the organic particulates in Louisville originates outside of Greater Louisville.[2]

The area-wide air pollution problem—the contamination of one city by an adjacent industrial complex—will be with us in the near future if not already here today. We can describe the weather conditions during which this may take place and then discuss some of the activities under progress to study them.

We think that there are three weather factors which favor air pollution episodes on a broad scale. First, small dilution due to light winds and a low altitude thermal inversion which inhibits upward mixing; second, the absence of rain scavenging, and third, the persistance of this weather for several days to permit the pollution to accumulate. This weather description fits the warm high pressure system. It is called a *warm* high because, unlike a polar mound of high pressure, the air within it is relatively warm in comparison to the surrounding atmosphere. In so far as I am aware, every important air pollution episode (Donora, London, and Meuse

Valley) has occurred with this weather condition. In Los Angeles, there is also a warm high pressure system present but other factors are also important. Orographic features and sources determine where and when an episode will occur during the presence of a warm high pressure system.

We can provide many illustrations of surface weather maps which are typical of a slow moving warm highs. Figure 1 shows the surface weather charts during four days of the famous Donora episode in October, 1948. Attention should be directed to the area of the middle Atlantic states and adjacent regions. The thin lines are isobars, the shaded area indicates precipitation. The relevant center of high pressure on the first day of the series is in southern Ohio. On successive days it jumps all over the eastern United States, a typical situation. The speed of the

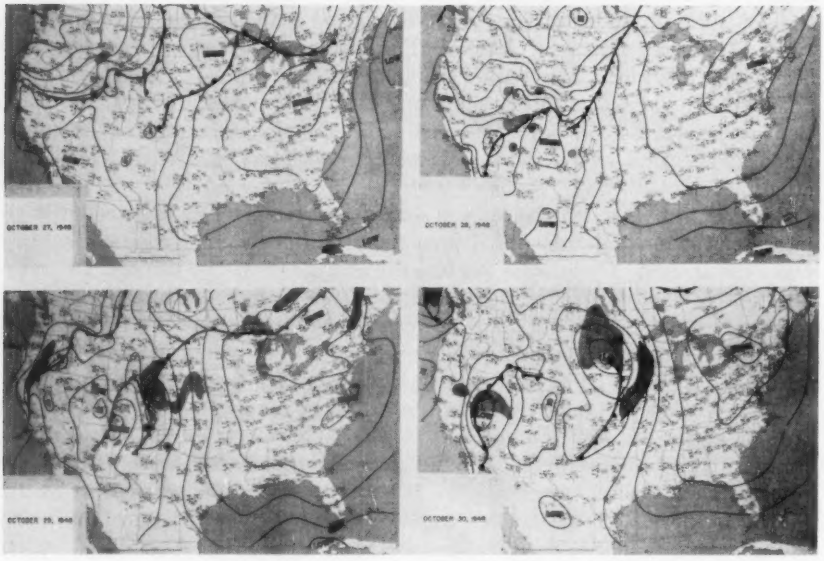

FIG. 1.—Weather maps during part of the Donora air pollution episode in October, 1948.

wind at the ground level and in the first few thousand feet of air is inversely proportional to the spacing of the isobars, more or less, on these maps. It is evident that the wide spacing associated with the region around the center of the high pressure results in very light winds. Local ventilation is severely restricted by this condition. Second, the air mass making up the high pressure is sinking. The result is a low level thermal inversion, with a base at about 3,000 feet in the afternoon and lower at other times. This inversion acts like a lid, preventing upward mixing. Because of clear skies the nocturnal outgoing radiation is intense, building up a very strong nighttime ground thermal inversion which likewise inhibits vertical mixing. In the London and Donora episodes, the air cooled enough to produce fogs which could not be burned off by the daytime solar heating.

When a large area of the United States is dominated by such a slowly moving high pressure system, the normal, fairly rapid, west-to-east air flow is replaced by air

which drifts about lazily and irregularly. Pollutants from one city can be added to those of another. The absence of vertical mixing limits all of the contamination to the layer of air near the ground.

Pack and Hosler[3] analyzed the weather situation during the passage of a warm high pressure center in October, 1956. To make the example more realistic, eight continuous sources were assumed. They had in mind continuous effluents from

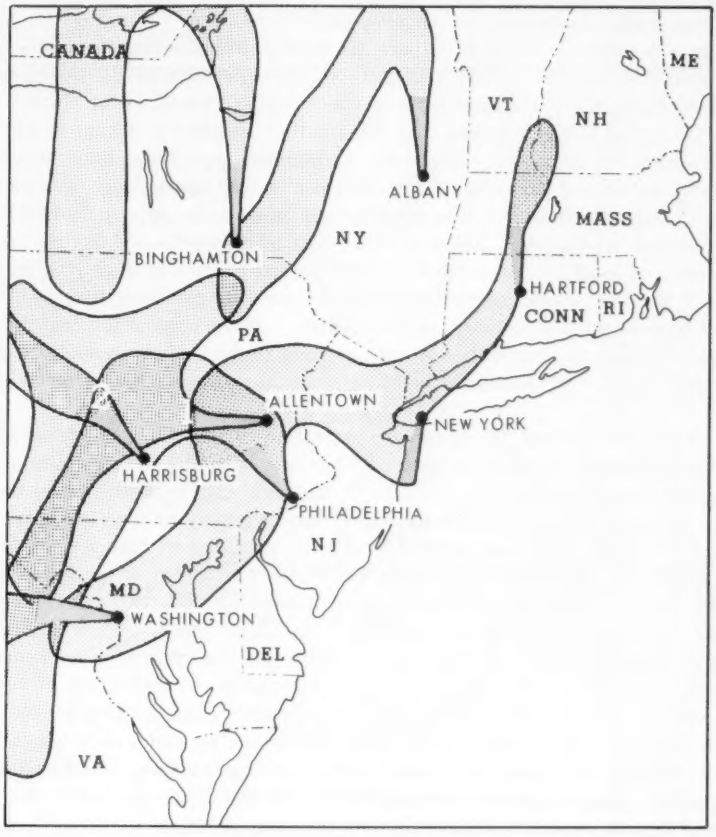

FIG. 2.—Area enclosed by 48-hour plume starting at 0500 G.C.T., October 11, 1956. Most recent 6 hour portion of effluent shown by heavier hatching.

reactors at eight locations. The number of industrial pollution sources would greatly exceed eight in the area which is shown in Figure 2. The plume is presumed to diffuse 11° each 6 hours of movement and mix upward to the top of the well-stirred daytime lower atmosphere. The shaded areas depict the regions covered by polution released from each of the cities, assuming the emission began 48 hours before the indicated time. The shading for the first 6 hours is more intense to distinguish it from the older more dilute clouds of pollutants. The irregular paths

are apparent. More important, however, is the overlap. In several places, the contamination from three sources contribute to the pollution. It is possible to compute average concentrations since the volume of air in each of these plumes can be readily estimated. The minimum volume which Pack and Hosler have derived from the weather data and their assumptions about spread and vertical mixing was about 10^{13} cubic meters into which all of the effluents released during a 48-hour period from one city are dumped. If the carbon monoxide released in the two days from, say, a metropolis like Los Angeles, 10,000 tons, were diffused in this air, an average concentration of about 1 part per million would result from this source alone over a wide area. While this may be only 1 per cent of the recommended threshold value, it is in the same range as concentrations found within cities.

We are trying to make certain that this weather situation is the one to which we should direct our attention. Area-wide contamination in warm highs was tested during the autumn of 1957 and 1958 by Weather Bureau meteorologists[4] at the Taft Sanitary Engineering Center who predicted its onset in the eastern United States. The National Air Sampling Network which normally operates one day during each two weeks, at random, was alerted to make special collections during these times. Each of the five special collections yielded dustloadings which consistently exceeded those of normal operation of the network. While most of the dust probably originated within the sampling city, this exercise shows the widespread occurrence of adverse weather conditions for pollution since many states were involved in each case.

We have also studied the radon concentration during the passage of a slow-moving warm high pressure system. Radon is a naturally occurring radioactive gas which is emitted from the ground. It has been amply demonstrated that the radon increases remarkably when atmospheric vertical mixing becomes weak. Thus, for example, nighttime ground level concentrations are higher than daytime concentrations. We selected to study the weather situation of the latter part of October, 1954. The measurements are available through the courtesy of Dr. Luther Lockhart of the Naval Research Laboratory and the observations were made in Washington, D.C. Figure 3 shows the diurnal cycle of radon concentration during three periods; first, during the passage of the warm high or so-called "stagnation" period; this having been determined before looking at the radon measurements. The average wind speed during the 89 hours of stagnation was only 4.7 knots. The other two traces show the average radon cycle before and after this period. The shaded area identified by "Poor Data" corresponds to the period of the daily filter changes while the radon decay products were not yet in equilibrium. The significantly higher concentration is apparent in the figure for the days during which the warm stagnant high lay over Washington. A further study of the radon observations may reveal other periods of potential widespread pollution which we otherwise may miss.

We have also studied the geographic frequency distribution of stagnant warm high pressure systems for the U.S. east of the Rockies.[5] The maximum frequency occurs in the southern Appalachian Mountains and decreases radially from this area. The analysis of cases by months reveals a pronounced maximum frequency in the month of October. The October geographical distribution is shown in Figure 4. The numbers on the figure denote the frequency during a 21-year period of

cases with four or more successive days of light winds (under 8 miles per hour). This October weather coincides with the pleasurable memories of Indian summers, periods of unusually fine weather after the heat of the summer has waned. We were somewhat surprised at the high frequency of cases, even in winter, when warm high pressure systems covered the southeast U.S. Some of these resulted from the extension of the Bermuda high pressure cell onshore, much further west than its normal position. It seems probable that with equal industralization, air pollution complaints would be more numerous in the southeast than, say, in New England with its stormier weather.

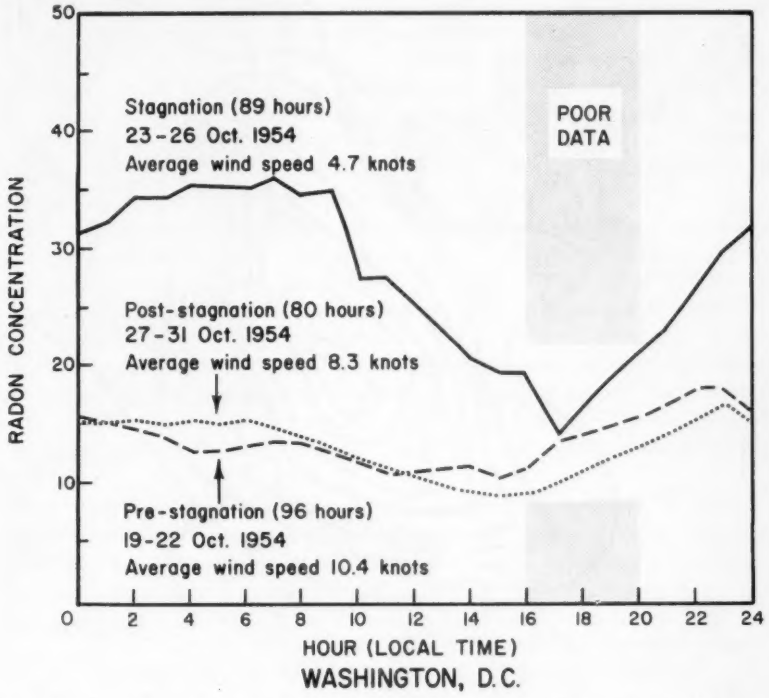

FIG. 3.—Diurnal course of ground level radon concentration showing the contrast between a period of light and normal winds.

I have tried to describe air pollution problems which may be with us in the future. The best way of avoiding them is by preventing the disposal of wastes into the atmosphere. If this future picture serves no other purpose than stimulating the sanitary engineer to take preventive measures, our wasted work will be justified. But if it still looks like air pollution is here to stay, there are several steps the meteorologist had better take; first, together with health officials, keep track of the atmosphere's cleanliness and second, be sure that he can adequately predict the dilution, transport, and removal by the atmosphere in case meteorological control becomes necessary, that is, if we must decide on the release times based on weather forecasts.

I suspect that in many ways the meteorologist is ahead of others in thinking about this broader scale of air pollution.

Tracer Studies.—The advantages of radioactive substances in atmospheric studies are at least two-fold. First, the possibility of detecting exceedingly low levels of radioactivity; for example, the radiochemist is able to measure one tritium atom in a billion billion hydrogen atoms in 1953 oceanic rain. Second, the difference in isotopic composition normally make slittle difference in the meteorological behavior

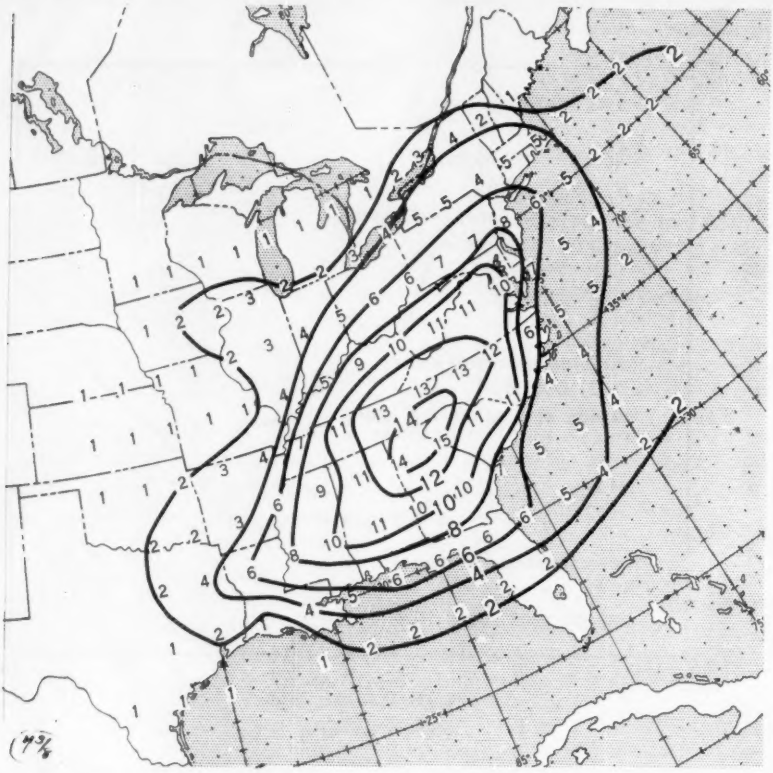

FIG. 4.—Number of periods of prolonged (4 days or more) light wind weather cases during 21 Octobers.

of the substance. Recently, for example, two Swiss scientists[6] dispersed silver iodide in cloud seeding experiments and marked it with radioiodine-131.

As we all are aware, today's radioactivity comes both from artificial and natural sources. A continuing test ban, while denying us fresh tracer material, will actually be to our advantage. The past tests have been so numerous that frequently the source of the measured fission-product radioactivity has been in doubt. Right now all of the fission products at ground level unquestionably come from the stratosphere since the tropospheric debris should have been effectively scavenged since October, 1958, the test moratorium.

Weapons-Created Radioactivity.—The stratosphere is the part of the atmosphere lying above the turbulent troposphere. It starts at about 35,000 feet in the polar and temperate zones and 55,000 feet in the tropical regions. The difficulty in placing observing platforms this high in the atmosphere, the absence of clouds and rain in the stratosphere, and the probable isolation of the stratosphere from the troposphere all have contributed to a previous lack of interest in stratospheric circulations. Now, however, we are able to measure winds regularly and to fly airplanes and missiles in much of the stratosphere with impunity. There is now reason to believe that the two layers are not independent of one another. Further, most of the solar weather effects, if there are any, are believed to influence tropospheric weather through their affect on the stratosphere.

There are certain kinds of information which the ordinary meteorological wind observations cannot provide in the stratosphere. These are the net bulk north-south transport—the meridional circulation which will be shown in a latter figure—and the diffusive mixing. The isolated meteorological winds are neither accurate enough nor sufficiently densely distributed to permit us to estimate these features. At present only tracer techniques are at our disposal.

Ideally the measurement of stratospheric radioactivity should be used to study stratospheric air movements. Unfortunately, the AEC strontium-90 (Sr^{90}) program is felt to be too poor for this purpose. Let me simply note, in passing, that the stratospheric sampling results are not at variance with what will be discussed below.

We do have confidence in the deposited stratospheric radioactivity over the globe. The details of the latest information were revealed during May, 1959, before the Joint Committee on Atomic Energy. Figure 5 is a plot, in the lower part of the figure, of the Sr^{90} fallout from the stratosphere plotted on a sine of the latitude scale.

The obvious features of this figure include the great stratospheric fallout in the north temperate zone, the minimum in the equatorial band, and a secondary maximum in the south temperate zone. Comparison with the rainfall profile, the upper part of the figure, eliminates rainfall amounts as the obvious explanation for the fallout profile. We also have ground level air concentration measurements whose north-south profile confirms this fallout picture. There seems little doubt that the stratospheric radioactivity finds itself located mainly in the temperate zones at ground levels.

The source of the stratospheric radioactivity prior to the spring of 1958 is largely from two locales: about 70 per cent from the U.S. atomic tests at 11°N and the remaining 30 per cent from the U.S.S.R. tests north of about 50°N. The contribution of these stratospheric inputs to ground level fallout depends on the time of the injections and the removal rate from the stratosphere. But as a reasonable approximation, it is likely that the fallout on the ground from these sources is very roughly in the same 70 to 30 ratio. This is because the U.S. Pacific injections were made earlier but left the stratosphere more slowly than the Russian injections.

The nuclear explosions at 11°N are sufficiently close to the equator that one might suppose equal partition between hemispheres if no special mechanism kept the hemispheres separate. We can test the hypothesis of equal partition between hemispheres from equatorial atomic debris by simple arithmetic. Assuming

that the Russian fission products remain entirely in the northern hemisphere, the ratio of northern to southern hemisphere fallout should be roughly 2 to 1, that is, 35 in each hemisphere from equatorial sources and an extra 30 in the northern hemisphere from the Soviet tests. The relative fallout ratio in Figure 5 is greater than this or about 3 to 1. Thus, not only has there been little transfer of temperate or polar stratospheric air into the southern hemisphere but about twice as much air at 11° N stays in the northern hemisphere.

What kind of a picture of circulation and mixing processes can explain these observations? It is my view, and that of many others, that the model suggested

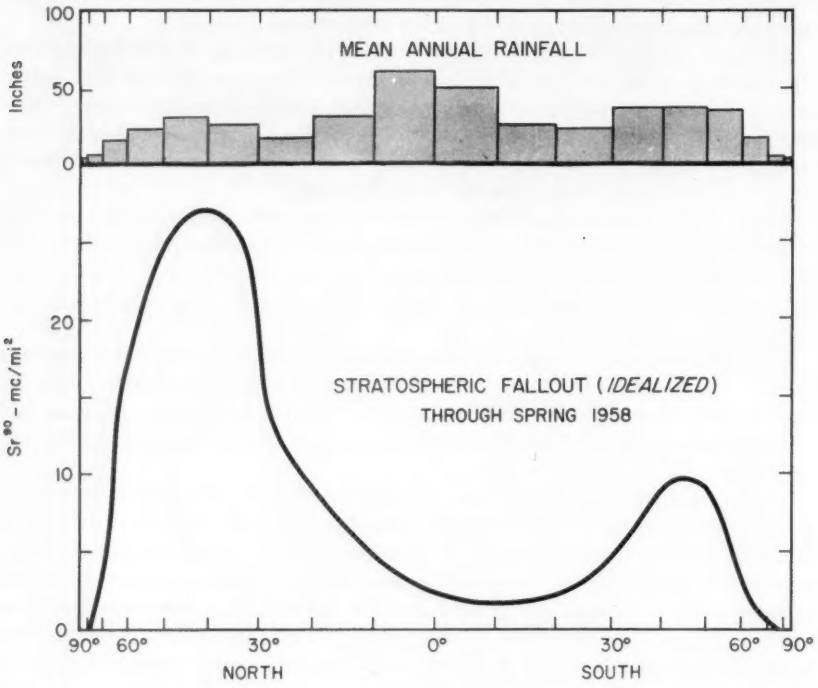

FIG. 5.—Latitudinal pattern of Strontium-90 fallout (lower part of figure) and mean annual rainfall in 10° bands (upper part of figure).

about 10 years ago by Drs. Brewer and Dobson[7] appears to be the best answer. As seen in the left side of Figure 6, the British have found exceedingly cold frostpoint temperatures in the stratospheric meterological research flights over England. Frost point temperatures of 190° Kelvin were found at 45 to 50,000 feet. Brewer and Dobson accounted for this dry air by passing it through the cold trap in the equatorial tropopause region. As is evident in the vertical cross section on the right, there are temperatures in this part of the upper troposphere and lower stratosphere which would condense water from an air mass rising through it to produce a frostpoint of from 190 to 200°A. Stewart[8] in England, as well as ourselves,[9] have embellished this idea with a complete circulation picture shown on the cross

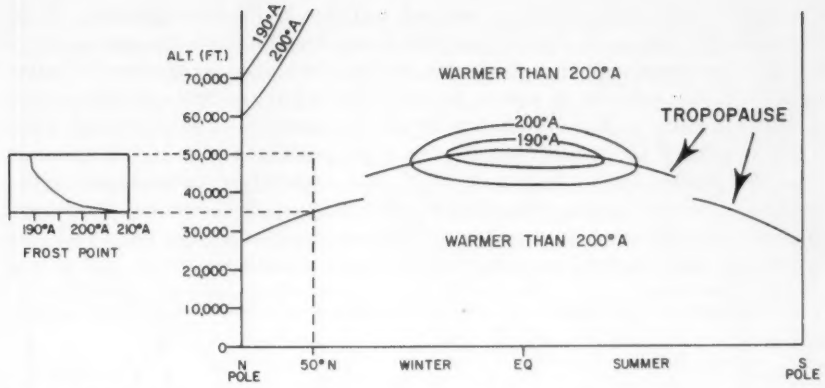

FIG. 6.—Schematic vertical cross section of the atmosphere showing tropopause and isotherms colder than 200°A (−73°C) and the frostpoint profile in the lower stratosphere over England.

section of Figure 7. A meridional circulation is proposed in which air enters the stratosphere in the equatorial region and exists poleward of the equatorial zone. Superimposed on this bulk transport is horizontal and vertical mixing which are probably much smaller than tropospheric values. Vertical mixing is probably very small in the stratosphere because of the marked thermal stability. From the fallout results, and from uncertain inferences from weather maps, even the horizontal coefficient of mixing is believed to be an order of magnitude less than in the troposphere. The meridional circulation determines the location of exit—the temperature or polar zones—and the unequal partition of air between hemispheres even for radioactivity injected near the equator. When the prediction of our version of the Brewer-Dobson model is compared with the fallout picture shown previously, the fit appears to us to support, but not prove, its correctness.

There is one other aspect of fallout observations which Brewer and Dobson also correctly anticipated. The thermal structure in the polar winter hemisphere and

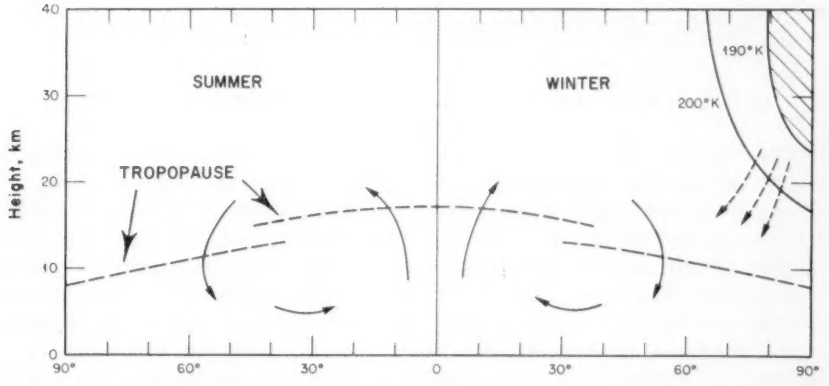

FIG. 7.—Atmospheric circulation model (after Dobson and Brewer).

the ozone observations suggest a seasonal variation in the sinking motion of the stratosphere. The main exit of stratospheric air should occur in the late winter or spring according to such reasoning. Figure 8 provides a history of deposited radioactivity in rain collected in pots or funnels which many countries are establishing to monitor fallout. The information during the period July, 1957, through June, 1958, is plotted by months, stratified into geographical regions. It is apparent that the greatest fallout (and most of it is of stratospheric origin based on the analysis of short-lived fission products) is in the spring and the minimum is in autumn. This parallels the seasonal ozone trend. The southern hemisphere temperate zone shows the same seasonal variation but with such a small amplitude that it is of

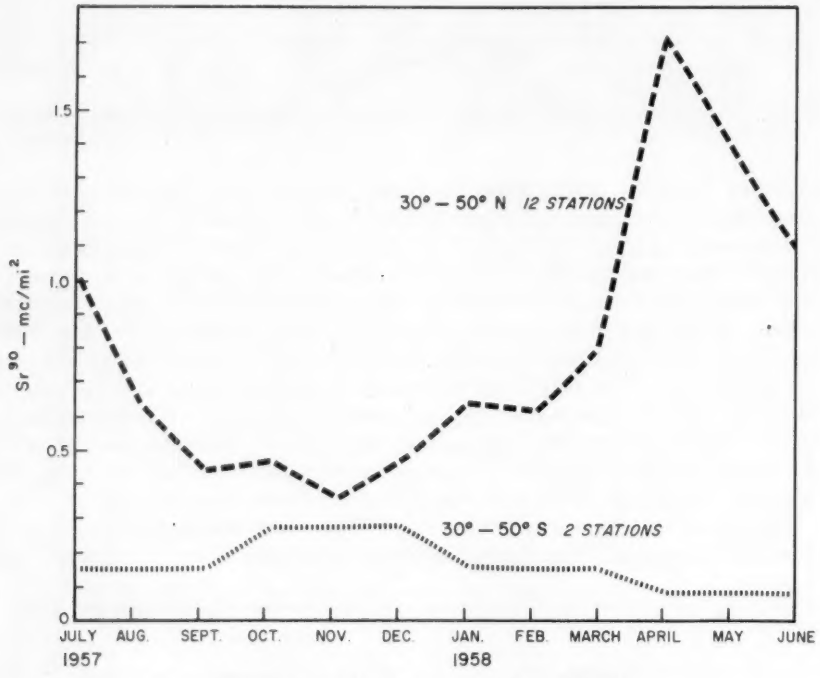

FIG. 8.—Average monthly deposition of Strontium-90.

doubtful reality. We should look to mechanisms in the late winter and spring to transfer air out of the stratosphere according to the radioactivity observations.

Two new tracer materials were added to the atmosphere during the U.S. Pacific tests in the summer of 1958. The first, rhodium-102, was created at very high altitude and can provide information on the exchange between the lower and upper stratosphere. The other is tungsten-185. This was added in both the troposphere and the stratosphere. A distinction in the source of the tungsten can be most readily made by studying the results many months after the tests have been completed. During this interval, it is believed that the material left in the troposphere will be effectively scavenged by precipitation. The preliminary results[10]

on January, 1959, six months following the addition of tungsten-185, are shown in Figure 9. The monthly ground level air concentration of stations along the 80th meridian are plotted against the latitude. The north-south profile exhibits an amazing similarity to the accumulated Sr^{90} given in Figure 5. Whereas there may be questions concerning many of the features in Figure 5, such as the separation of the Sr^{90} deposition into tropospheric and stratospheric components and the relative source of the Sr^{90} from equatorial or polar latitudes, these ambiguities are not present in Figure 9. The source of the tungsten-185 is the equatorial stratosphere.

The Russians, on their part, added so large an amount of fission products to the

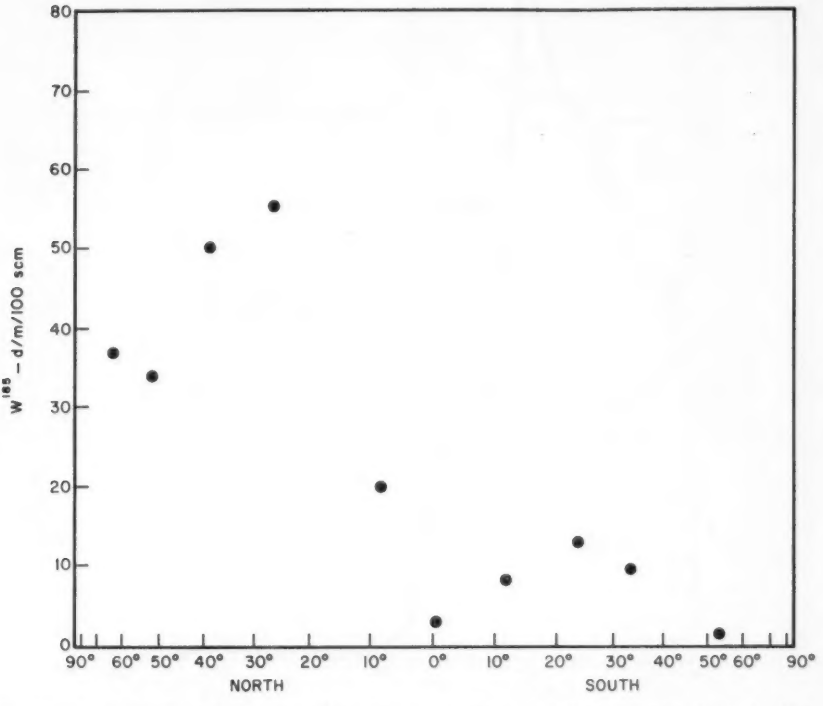

FIG. 9.—Latitudinal profile of ground level air concentration of Tungsten-185 along the 80th meridian in January, 1959.

polar stratosphere in October, 1958, that one should have little difficulty in following its history both in the stratosphere and in ground level fallout. A limited amount of stratospheric evidence suggests that for the first new months following the Russian injection very little material was carried as far south as 45°N (the injection took place at 73°N). On the other hand, the fallout in the north temperate zone has responded remarkably to the enhanced stratospheric content. For example, Figure 10 plots the north-south profile of ground level air concentration of gross fission products in April, 1958, and April, 1959, from the Naval Research Laboratory's 80th meridian network. As is evident from the figure, six months

after the October 1958 injection, the concentration of stratospheric fission products in the ground level air in 1959 greatly exceeds the total air concentration in 1958. But the increase is exclusively limited to the northern hemisphere, suggesting that on a time scale of a half year little or no Russian debris has found its way into the southern hemisphere stratosphere. This is consistent with the Brewer-Dobson type of circulation.

Time does not permit a review of the many other meteorological problems which are being studied by fission-product tracers: tropospheric mixing, trajectory veri-

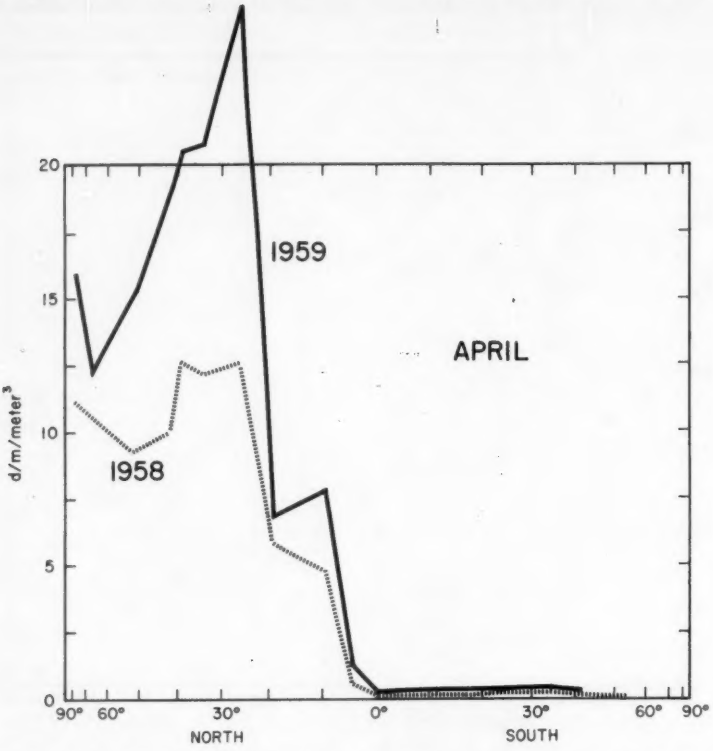

FIG. 10.—Latitudinal profile of ground level air concentration of fission products along the 80th meridian in April, 1959. Dashed curve shows the same profile in April, 1958.

fication, rain scavenging, the hydrologic cycle using tritium, and many others. In the last few moments I would like to discuss an application of cosmic ray produced radionuclides.

Cosmic Ray Radioactivity.[12]—A list of the known cosmic-ray produced radionuclides is given in Table 1 along with their decay products. Several of these radioisotopes (carbon-14, tritium, and possibly sulfur-35) are also created during nuclear testing.

We will consider only the isotopes with half-lives of tens of days, phosphorous-32 and 33 and beryllium-7 which lie in the range of times of atmospheric processes.

TABLE 1

Radioisotope	Half-Life
Be^{11}	2.7×10^6 years
C^{14}	5700 years
H^3	12.5 years
S^{35}	87 days
Be^7	53 days
p^{33}	25 days
p^{32}	14.3 days

They are particulate and probably attach themselves to natural aerosols of the same size.

The idiosyncracies of rain scavenging complicate the interpretation of these radionuclides when they are collected in rain. The content of rainwater may be

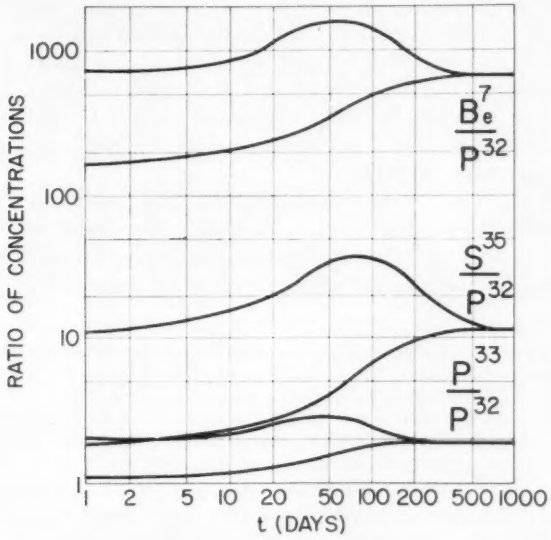

FIG. 11.—Ratios of cosmic ray produced radionuclides which can be found in the troposphere. For lower branches of the curves abscissa is irradiation time (air contains no radionuclides at $t = 0$). For upper branch of curve abscissa is time after entrance of stratospheric air into troposphere.

low either because of previous scavenging of the air mass or because of, say, ineffective scavenging of dust. To overcome this kind of ambiguity, Peters has suggested the use of ratios of isotopes which are independent of the character of the rain removal process.

The history of these ratios is shown in Figure 11. The lower branches of the curves pertain to air masses which gain radioactivity in the troposphere after being completely free of the radionuclides at $t = 0$. The upper branches refer to air which descended into the troposphere after its radioactivity had come into equilibrium with the cosmic ray radiation of an intensity equal to that encountered slightly above the tropopause. The differences in ratios between air of strato-

spheric or tropospheric origin is sufficiently marked to be able to distinguish the source. Thus, a beryllium-7 to phosphorus-32 ratio of the order of one hundred would reflect the absence of stratospheric air. On the other hand, ratios of the order of four hundred would indicate that the air mass recently came from the stratosphere.

The Indians have made a number of measurements of this ratio in the rains at Bombay (19°N) and Kodaikanal (10°N). No ratio exceeded 290 and the average was about 120. This indicates the absence of stratospheric air in the rain levels over tropical stations agreeing with the Brewer-Dobson model and almost every other concept of global circulation. It would be much more valuable to obtain similar information for temperate or polar latitudes, particularly in the spring when the Brewer-Dobson model would predict an exit from the stratosphere.

This application of natural radioactivity is only one of many which are available to meteorologists. There is much to be gained from observations of radon and its decay products in understanding tropospheric transport and mixing, air mass identification, air pollution studies (beyond that mentioned earlier), and many other fields. Unlike the intricate chemistry required to identify the minute amounts of cosmic ray radionuclides, the tools for measuring the radon and thoron decay products are universally available.

Conclusion.—The tools of the nuclear physicist and chemist are now available to the geophysicist and it is up to him to use them. This article has reviewed only two of the applications. In time it is predicted that many others will be employed and that the measurements of radioactivity will be commonplace in meteorological research.

[1] Personal communication from Dr. C. D. Keeling, Scripps Institution of Oceanography.

[2] Personal communication from Mr. R. McCormick, U.S. Weather Bureau, assigned to the Public Health Service Taft Sanitary Engineering Center in Cincinnati, Ohio.

[3] Pack, D. H., and C. R. Hosler, "A Meteorological Study of Potential Atmospheric Contamination from Multiple Nuclear Sites," *Proceedings of the Second United Nations International Conference on the Peaceful Uses of Atomic Energy*, **18**, 265 (1958).

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[12] This section is based on Peters, B., *Jour. Atmos. Terr. Physics*, **13**, 351 (1959).

PROBLEMS IN BIOCLIMATOLOGY

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Ideally, the bioclimatologist should have the characteristics of both the classical and the romantic type of scientist. For he must deal quantitatively with the measurable effects that the known forces of the physical environment exert on biological processes. And he must also cultivate an awareness of the fact that other undefined cosmic factors influence in obscure but profound ways the growth, behavior, and fate of all living things. I must acknowledge immediately that I have never worked in any aspect of bioclimatology, nor have I made a systematic survey of the relevant literature. But as a student of the etiology of disease, both in individuals and in complex populations, I have come to realize, like many others, that bioclimatological mechanisms often condition both the etiology and the manifestations of pathological processes. While this type of experience constitutes no justification for dogmatic statements on bioclimatological problems, it has led to more general questions regarding the effects that environmental forces exert on living things and particularly on man. These questions I shall now try to formulate.

Biological Rhythms.—As every one knows, most biological phenomena exhibit rhythms which are linked to those of the physical world. There are many well documented examples of biological cycles characterized by daily, seasonal, annual, or longer periodicities, and some of them have been studied in the laboratory with exquisite precision. For example, the phototactic response of Euglena exhibits a rhythm with a 24 hour period which is independent of temperature, at least between 16°C and 33°C. The fact that this endogenous rhythm is exhibited by a unicellular organism demonstrates that "biological clocks"* do not require the complexities of nervous organization.[1] Other phenomena like the emergence of insects into activity act as landmarks for the season of the year and may be more complex in their determinism.[2] Our colleague, Dr. Frank L. Horsfall, has told me that he shelters under his Long Island home a colony of termites which regularly emerge between March 15 and March 25 every year, independently of any climatic factor of which he is aware.

Clearly, these rhythms are the manifestations of built-in biological clocks and for this reason they appear at first sight to have no bearing on our symposium. After all, as pointed out by Dr. Konrad J. K. Buettner,[3] nearly all biological and meteorological factors have a yearly and a daily period and, therefore, any correlation between the two groups is but an expression of the fact that weather and life take place on a revolving and rotating earth. Nevertheless, the problem of cycles is one pertinent to our discussion because the biological clocks are not as immutably set as appears; instead, they rapidly change their timing in accordance with changes in the physical environment. Let me illustrate this statement with a specific example taken from a very recent publication.

In man, the urinary excretion of 17 hydroxycorticosteroids exhibits a well-defined and fairly stable daily rhythm. Thus, measurements of these adrenal hormones made at very frequent intervals during a thirty-hour shift by air travel from Continental United States (Central Standard Time) to Japan and Korea, revealed that the urinary excretion remained synchronized with C.S.T. even after arrival in Asia.[4] Progressively, however, the timing of excretion changed and after 9 days it had be-

TIME DISPLACEMENT OF POTASSIUM URINARY EXCRETION

Place	Date	Meq/3 hr. at indicated time*		
		0–3	9–12	21–24
U.S.A.	8/1	3.1	15.8	5.7
Japan	8/5	21.8	6.5	11.4
Korea	10/14	4.5	12.7	4.6

* Time is recorded as local time.
(Data from E. B. Flink and R. P. Doe, 1959).

come synchronized with Asian time. The rhythm of excretion of sodium and potassium exhibited a similar pattern. Likewise, other physiological phenomena have cyclical patterns which are under the influence of the environment. Thus, the diurnal temperature rhythm in man was observed to change following airplane flight from Ontario to England. In this case, it took three to four days for the Canadian temperature rhythm to fall in step with the European rhythm.[5] The problem of biological cycles certainly lends itself to experimental analysis since changes in rhythm can be produced at will in laboratory animals. For instance, it has been possible by inverting the light schedule of mice for two weeks to produce shifts in daily rhythm with regard to blood eosinophils, mitoses in pinnal epidermis of liver, he patic nucleic acid metabolism, and blood levels of corticosterone.[6]

As is well known, the Hippocratic writings repeatedly and forcefully emphasized that the occurrence of many types of disease has a marked seasonal character. In our communities, every one is aware of the winter incidence of acute respiratory disease and the summer incidence of poliomyelitis among human beings. And our colleague, Dr. Richard Shope, never tires of discussing the striking autumn incidence of hog influenza and hog cholera among the swine herds in the Middle West. Less well known, but almost as pronounced, are the seasonal ebbs and flows in the clinical manifestation of metabolic disorders, for example diabetes and circulatory diseases.[7, 8]

A number of well known facts immediately come to mind to suggest mechanisms through which climatological factors could indirectly affect the incidence or severity of disease. Crowding, physical activity, availability of certain types of food, prevalence of parasites and their vectors, etc. etc., are all factors in the causation of disease which are profoundly conditioned by the physical environment. But in addition to these obvious determinants there are others less well recognized, which are probably more significant. This belief is based on the fact that the internal environment of man—as well as of animals—is more variable than was believed a generation ago.[9]

There is no doubt of course that the essential characteristics of the internal environment must remain within certain limits to be compatible with the maintenance of life. On the other hand, it is also true that some of the biochemical activities of tissues can undergo profound quantitative variations and that some of these changes exhibit a marked seasonal pattern. A striking illustration of these biochemical cycles was discovered by C. and G. Cori some 30 years ago.[10] In the course of their studies on sugar metabolism, the Coris became aware of a marked seasonal variation in the ketonuria of rats kept without food for 48 hours. During the summer months (from May to October) the excretion of acetone bodies brought about by fasting proved consistently to be three times greater than during the winter months. The fact that the excretion of acetone bodies did not rise during the winter when the rats

SEASONAL KETONURIA IN FASTING RATS*

Month	Mg of acetone bodies per diem per 100 gm. wt.	per rat
Apr.		32
May		67
June	6.2	51
July		36
Sept.		12
Oct.		5
Nov.		7
Dec.	1.9	5
Jan.		12
Feb.		7
March		7

* Data from G. and C. Cori, 1927 (seasonal averages) and from Burn and Ling, 1928 (monthly averages).

were placed in a room at a temperature comparable to that of the summer provides evidence that factors other than heat were responsible for the greatest fasting ketosis observed during the summer.

These findings have been confirmed and extended in England by Burn and Ling,[11] who found indeed that the difference in fasting ketonuria between the spring-summer season and the fall-winter season was even much greater than that observed by the Coris. With the strain of rats used in England, the difference was of the order of ten-fold. Furthermore, the amount of glycogen in the liver of rats after 24 hours' fat diet also proved to vary according to a seasonal pattern, being much higher in the winter than in the summer.

There have been suggestions that this seasonal change has an evolutionary basis, namely that animal tissues have developed mechanisms which enable them to withstand successfully long periods of starvation in the winter. According to this view, energy requirements during the winter would be more likely to be met by combustion of the fat stores whereas this metabolic mechanism would not play as essential a role during the summer.[9, 11, 12]

While the intimate biochemical processes involved in the shift from summer to winter metabolism need not be discussed here, it is of interest to point out that the summer ketosis was associated with a reduced capacity of the tissues to oxidize glucose, and was probably due to a reduced functional activity of the pancreas. It appears, in other words, that the seasonal patterns of physiological behavior can have their basis in seasonal variations of hormonal activity. There are, of course, many other examples of cycles involving hormonal activity—for example, those associated with menstruation or those resulting in the diurnal variation in output of adrenal corticosteroids mentioned above. What must be emphasized anew at this time is that these built-in cycles are influenced by variable climatologic factors. It has long been known that the size of the thyroid and of the adrenals is normally greater in the winter than in the summer in laboratory animals, and can be altered at will by changing the environmental temperature (for a recent example, see reference 13). We have seen also that the rhythm in secretion of adrenal corticosteroids progressively varies when the geographical environment is changed. In fact, the study of these effects constitutes a rapidly expanding field of animal physiology. Suffice it to mention here as examples the studies of human performance in high mountains[14, 15] and of the nutritional aspects of climatic stress.[16] Professor Alexander von Muralt has kindly provided me for this occasion with a list of papers

dealing with the pathological effects of weather on man. From these studies it appears that objective tests are available for quantitative observations as shown by the fact that warm fronts are associated with a decrease, and cold fronts with an increase, in capillary resistance.[17] It can hardly be doubted therefore that disease states—which in final analysis are always the expression of physiological disturbances—can be affected by the complex of physical forces which make up the climatological environment. Moreover, this statement applies not only to metabolic disorders, but just as well to diseases caused by microbial agents.

Population Fluctuations.—In addition to diurnal and seasonal rhythms which are well documented, there are other biological cycles with longer periodicities. It has long been known, of course, that plant and animal populations in the wild undergo tremendous quantitative changes.[18] For example, the records of the Hudson Bay Company provide fascinating material to document the statement that there have occurred large fluctuations in the numbers of fur animals as well as of the rodents on which they feed.[19, 20] The analysis of historical records and of the findings in recent wild life surveys have led to the belief that many population changes are determined by climatic factors, and furthermore there has been a tendency to accept that some at least of these changes exhibit a cyclic character. While the evidence for a true periodicity is not always convincing, there seems to be little doubt that population fluctuations are often the expression of responses to changes in the physical environment.

Well documented information bearing on this problem has come from the study of tree rings in the North American continent. Comparison of the thickness of tree rings has revealed that marked changes have occurred in the rate of plant growth during the past 2,000 years, probably as a result of variations in temperature and in atmospheric precipitation. There is reason to believe that these changes have also played an important part in the life of the Pueblo Indians—affecting the location of their settlements and the size of their population.[21] It is worth mentioning here that, as repeatedly emphasized by Huntington, climatic changes have probably been influential also in determining the growth and decay of other civilizations all over the world.

Fairly accurate information derived from wild life surveys in our time has provided evidence that the climate conditions both the distribution and the abundance of several animal species—as illustrated by the history of rabbit populations in Australia.[22, 23] The European rabbit *Oryctolagus cuniculus* was introduced into Tasmania at the beginning of the 19th century and spread over much of the island. Very rapidly, color variants became established and they now exist with different frequencies in different areas—the black rabbits reaching a frequency of 20 per cent in places of highest rainfall. This particular example is of special interest because it illustrates that climatic factors can operate through genetic mechanisms. A related example is provided by the well-known fact that animals living in colder climates are usually larger than those of related species living in warmer climates. In most situations considered in the present report, however, mechanisms other than genetic must be invoked since the biological responses to climatic changes occur so rapidly that they can hardly be due to genetic alterations.

In certain cases, the explanation appears rather straightforward, for example with regard to plankton which changes continuously in abundance and composition from

season to season and from year to year. In 1925 the warm equatorial counter-current off Columbia and Ecuador (El Nino) shifted its course so strongly to the South (as it does once every seven years) that the population of plankton, fish, and water birds normally found off the Peruvian coast fled or died, being replaced by warm water species.[24] When the current from the Atlantic predominates on the English coast, it brings water rich in phosphate which favors one species of glassworm on which the herring feeds. In contrast, current from the Channel brings in water poor in phosphate, resulting in failure of the herring fishery. The "red tide" which swept immense numbers of dead fish into the Florida beaches in 1946 and again in 1952, as it does approximately once a decade, was caused by a microscopic flagellate which is always present in the waters off the Florida coast, but in numbers too small to be harmful; its population reaches toxic levels only when atmospheric circumstances bring about the local stagnation of low-salt brackish water in certain areas.

In contrast to these fairly simple situations, the biological findings remain unexplained in most cases. Over the past three decades Errington[25] has analyzed the wild life surveys in Iowa with regard to populations of the ruffed grouse, the snowshoe hare, and especially the muskrats. The results of his analysis leave no doubt that the numbers of these animals have fluctuated enormously during the period under consideration. Yet there is no indication that heat, humidity, water levels, and other obvious variables can account for population changes, for disease states, or for patterns of behavior of the animals. While the findings are not explainable in terms of the meteorological data customarily recorded by the Weather Bureau, it is not impossible according to Errington that the biological patterns are related to the intensity of ultraviolet or other radiation—perhaps indirectly through some effect on the qualitative characteristics of the food available to the animals.

Decreases in the numbers of wild animals are commonly associated with a variety of disease states—both of metabolic and infectious character.[25, 26] It is therefore of importance to inquire into the evidence that climatological factors can actually affect resistance to disease. In fact, as already mentioned, there is a widespread belief among lay persons and physicians alike that certain types of weather disturbances are associated with particular illnesses.

In this country, Petersen and Mills[7, 8] have long emphasized that the incidence and gravity of each type of pathological disorder can be correlated with either climate or weather. As illustration it will need suffice to mention here two types of weather which appear to be potentially harmful to man. One is the frontal or disturbed weather, the other the Föhn or Chinook with descending subtropical air in the whole troposphere, each type apparently bringing in its train a specific set of clinical and physiological events. Most familiar is the conviction expressed in many folklores that pains from scars and from arthritis sharpen during weather of the frontal type. Few are the persons indeed who do not believe that:

> "A coming storm our shooting corns presage, Our aches will throb, our hollow tooth will rage."

There are many reports, on the other hand, that periods of Föhn in Switzerland and in Southern Germany are associated with increases in death rates, in automobile accidents, and in circulatory as well as mental disorders.[3]

In these special climatic situations, the pathological disorders seem to occur with-

out any detectable change in any of the known geophysical surface elements. The patients may not even be aware of any bad weather in the usual sense. It would appear, therefore, that these weather disturbances operate through physical factors which are still obscure or even completely unrecognized. A few related observations made with microorganisms are worth mentioning at this time. Whereas the metabolism of bacteria and yeast seems to be attenuated during cyclonic periods, it is intensified during anticyclones. By recording automatically such activities as luminescence, motility, acid production, sporulation, etc., it was found that the changes occurred so rapidly ($^1/_2$–1 hour) that they could not be correlated with the usual daily weather curves.[27]

Geophysical Factors Involved in Bioclimatology.—It would be appropriate at this point to discuss in detail the specific components of climate which are known, or have been claimed, to exert biological effects. However, this aspect of the problem will be treated cursorily because of shortness of time and even more for lack of convincing knowledge.

Temperature and humidity are of course the two climatological factors which are best understood. At their simplest their biological effects are illustrated by the close connection that exists between air temperature and the tempo of a cricket's chirp. It is said that counting the number of chirps in 14 seconds and adding 40 will give the temperature within a couple of degrees. Likewise, the higher the temperature, the faster ants move. As example of more complex effects of temperature, one could quote the discovery by Dr. André Lwoff (reported at the present meeting of the Academy by Dr. Albert Sabin) that a difference of 2°C can bring about the selection of virulent or avirulent mutants of polioviruses.[28] On the other hand, temperature and humidity have also less direct consequences by reason of the physiological responses that they elicit in living things. In the case of man, his semitropical origin is reflected in the narrow range of atmospheric environment to which he is adapted in his native biological state. Any departure from this environment is likely to cause physiological disturbances. A temperature of 29.4°C (85°F), with moderate humidity and low air movement, seems best for human comfort in the absence of housing and clothing. In practice, these artificial aids supplement several physiological mechanisms which permit a fairly wide range of adaptive heat control. Thus, enormous changes in blood flow through the skin capillaries can occur within a few minutes and regulate heat loss upward or downward as needed. When increased blood flow proves inadequate for rapid cooling, the sweating mechanism comes into play and provides heat loss by evaporation. While the needs for temperature control are more prolonged, for example in cases of passage from one season or one country to another, the body can regulate its own heat production through changes in metabolic rates. Needless to say, these regulatory mechanisms are effective only within a limited range and, furthermore, any excessive demand on them will cause profound physiological disturbances. In fact, as already mentioned, there is an enormous amount of clinical evidence that weather changes are commonly associated with exacerbation of many disease states. As Hippocrates said 2,000 years ago, "It is changes that are chiefly responsible for diseases, especially the greatest changes, the violent alterations both in the seasons and in other things. But seasons which come on gradually are the safest, as are gradual changes of regimen and temperature."

Contrary to common belief, it has not yet been shown that pressure changes *per se* can affect either the comfort or the health of man—except of course in the special cases of life at great depths or high altitudes. It may be worth mentioning at this time, however, that very slight reductions in pressure have been shown to exert profound effects on insect behavior, effects which appear to be independent of oxygen tension. In the laboratory, as well as in the field, the feeding habits, rate of development, and locomotor activity of higher insects are appreciably increased by slightly lowered or falling pressures. These conditions also seem to be associated with the sudden occurrence of mass emergencies.[29]

Needless to say, there is no general statement that can serve to describe the multifarious biological effects of the various types of radiation. Their deleterious effects go from reversible lesions in the skin to the production of lethal hereditary defects. Their beneficial effects range all the way from the synthesis of vitamin D to the orientation provided by polarized light for the motion of insects. The use of artificial light to prolong and increase egg production denotes profound influences on the endocrine system, and this becomes manifest also in bird migrations and in many other complex biological processes.

Recently, experiments with cosmic rays and their secondaries have pointed to the existence of even more profound hormonal effects of radiation. Whereas no clear evidence has been obtained that ordinary cosmic rays have any biological activity, cosmic ray shower electrons (produced by cosmic ray particles that penetrate into heavy matter) were found to increase mutation rates in a fungus, to interfere with normal reproduction in rabbits, and to accelerate the rate of development of cancers in mice pretreated with 20-methylcholantrene.[30]

Among other climatological factors which have been recognized recently are the small ionized molecules of the air and the so-called "sferics" which stem from natural electric discharges. It has been claimed that positive space charges have deleterious effects whereas negative space charges have beneficial effects—as illustrated, for example, by enhancement of proliferation of tissue cells exposed *in vitro* to negative ions.[31] Even human patients have apparently benefitted from such treatment.[3] If these claims can be validated, they point to a neglected aspect of biophysics, namely the space charges of small ions.

Air pollution is a bioclimatological factor of increasing importance in the causation of disease. Air pollutants range in kind from pollens and other allergens to toxic gases and aerosols produced by industrial plants, automobile exhausts, and domestic fires. Free HCl and H_2SO_4, sulfur dioxide, nitrogen dioxide, ozone, hydrocarbons, and pulverized rubber from automobile tires are but a few of the air pollutants known to exert toxic effects on human, animal, plant, and microbial life.[32, 33] The disappearance of lichens from modern cities, and the tremendous toll exacted by chronic bronchitis in certain industrial areas, serve to illustrate the varied aspects and the magnitude of the problem. Ozone deserves to be singled out in this discussion because it is present in large concentration in the smogs over Los Angeles, Phoenix, and Tucson, as well as in the atmosphere reached by high altitude flying. Even short exposure to the concentrations of ozone encountered in these circumstances produces pulmonary oedema and increases the susceptibility of experimental animals to bacterial infections.[34–36] It is worth mentioning in this respect that in Switzerland the Föhn seems to bring down large amounts of ozone from the upper

atmosphere, a peculiarity which has been claimed to account for some of the untoward physiological effects of this type of air current.[3]

Air Conditioning.—It is theoretically possible to control almost any factor of the indoor environment—temperature, humidity, pressure, radiation, space charge, size and composition of aerosol particles, etc. And it is obvious that air conditioning has already greatly contributed to general comfort, relief of allergic symptoms, and increase in working efficiency. At first sight, therefore, it would appear that control of the indoor environment is always desirable and that the only practical problems that it presents are those to be dealt with by architects and engineers. In reality, however, air conditioning has biological implications that transcent comfort and working efficiency, and that are still obscure.

While it is easy to appreciate the immediate direct effects of air conditioning, such as the sense of well-being and renewed vigor, it is difficult to predict its distant and indirect effects. Little is known, for example, concerning the responses of the mucous membranes and of the vascular bed to sudden and repeated shifts from the hot humid atmosphere of the street to the cool and dry environment indoors. Comfort of the moment may have to be paid for in the future in the form of new respiratory and circulatory disorders.

More studies are also needed with regard to the effect of air conditioning on the ability of the body to adapt to unpleasant and even dangerous environmental factors. To illustrate the range of these adaptive processes, it will suffice to mention again the physiological mechanisms used by the body to regulate its temperature, and to refer to the new finding that progressive exposure to low concentrations of ozone increases resistance to the toxic effects of this gas.[37]

Even more important perhaps is the fact that air conditioning may interfere with some of the diurnal and seasonal cycles discussed earlier in this report. One needs only recall here the variations in endocrine activities that are brought about by changes in temperature or in exposure to light. Of interest also are the claims that an increased rate of growth appears to result from the removal of certain metabolic stresses.[38] No information is available concerning the effect of air conditioning on processes which are so obviously correlated with seasonal metabolic changes. Yet these problems will certainly become of increasing urgency as man achieves greater control over his physical environment and removes himself further from the physicochemical conditions under which he has evolved as a physiological machine.

Conclusions.—I have tried to illustrate in this essay some of the effects of climatological factors on different types of biological phenomena: on the regular endogenous rhythms which exhibit diurnal or seasonal periodicities; on the long range fluctuations in size and behavior of populations; on the immediate physiological disturbances which are caused by the vagaries of the weather. I have also tried to emphasize that the biological responses to the environment cannot be described merely in terms of immediate direct effects, but must be regarded as dynamic processes, conditioned by the adaptive powers of the organisms, and often resulting in long range cumulative alterations. More than anyone, I realize the superficiality of my knowledge in these fields. Nevertheless, I cannot help expressing my belief that living things, including man, respond not only to heat, humidity, light and other obvious climatic components which are readily perceived by the senses, but also to many other environmental factors not readily identified, and in part still unknown.

Awareness of these complexities may not be helpful in solving practical problems. But it teaches humility to the overconfident biologist. In this spirit I had intended to close this essay with Hamlet's words:

> "There are more things in heaven and earth, Horatio, Than are dreamt of in your philosophy."

But the wise remarks of one of my critical colleagues now seem more apropos. In his words: "A lack of correlation with known environmental factors does not prove that unknown factors are operating. Primitive man explains natural phenomena with magic; when we are confronted with the inexplicable we too often fall back on mysticism, in somewhat more sophisticated language. This is a matter of personal taste, and on this point I must confess to being rather more with Horatio than Hamlet. Horatio no doubt was limited in his philosophy, but Hamlet believed in ghosts."

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*THE REACTIVITY OF A NATURALLY OCCURRING QUINONE (Q-255) IN PHOTOCHEMICAL REACTIONS OF ISOLATED CHLOROPLASTS**

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In a recent paper we showed that vitamin K derivatives restored photochemical activity to chloroplasts which had been extracted with petroleum ether. Since the petroleum ether extracts contain mainly carotenoids, Lynch and French[1] initially attributed the effect to β-carotene, but we observed that purified β-carotene was completely inert.[2, 3] Vitamin K and other typical fat-soluble vitamins could be expected to be removed by the extraction process, and since vitamin K has often been reported to be localized in the chloroplasts, it was logical to assume that this might be the active substance extracted. Furthermore, vitamin K is an effective cofactor for the photophosphorylation catalyzed by chloroplasts.[4–7]

Added vitamins K_3 and K_5 gave the anticipated activation effect[2, 3] and therefore we assumed, following the literature, that vitamin K was present in the chloroplasts and that it was the natural reactivating substance removed along with the carotenoids and other lipid-like substances. Since then, we have made analyses of the petroleum ether extracts and of the total pigment content of the chloroplasts. To our surprise, we found little, if any, vitamin K. In its place we obtained a crystalline, yellow compound which can be reversibly oxidized and reduced.[8] Its color and spectral behavior turned out to be identical with a substance extracted recently by Crane from alfalfa,[9, 10] which belongs to a newly-discovered group of "ubiquinones." Crane *et al.*,[9, 10] as well as Folkers and colleagues,[11] have obtained suf-

ficient information to characterize this compound as a substituted *p*-benzoquinone: 2,3-dimethyl-5-(3′methyl-2′ butynyl-oktakis-(3′-methyl-2′ butynylene)) benzoquinone.

We have now extracted from the chloroplasts of spinach, sugar beet, and swiss chard sufficient quantities of this material to test its ability to restore the photochemical activity of petroleum ether-extracted chloroplasts. Qualitatively the effect of the new benzoquinone is the same as that of the naphthoquinones (vitamin K, etc.). Quantitatively it is much more effective and, so far, the only compound which produces the same or even better rates of the photochemical reaction than the unextracted controls.

Materials and Methods.—The methods for preparing, lyophilizing, and extracting the chloroplasts, the method for determining the photochemical activity (with potassium ferricyanide as oxidant), and the method for the re-addition of various compounds to extracted chloroplasts were the same as previously described.[2, 3] In addition, we have used dichlorophenol-indophenol to compare the reactivation of the photochemical activity with two Hill oxidants of different characteristics. The reduction of this dye was followed spectrophotometrically after the method of Lynch and French.

The petroleum ether for extraction and chromatography was first treated with sulfuric acid, washed several times with water, dried with anhydrous sodium sulfate, and finally distilled; the fraction going over between 30°C and 40°C was collected and used for extractions. Other solvents were of sufficiently high purity to exclude contaminants which would interfere with the determination of absorption characteristics in the ultraviolet region.

The first step in the fractionation of the extracts was the same as previously described. The powdered sugar column effectively removes the chlorophylls and xanthophylls contained in the extract, and the fraction that comes through the column contains β-carotene and the other lipid-like compounds. It is this fraction which, as previously shown, contains the component(s) responsible for reactivation of chloroplasts rendered inactive by extraction.

For further fractionation and purification, this yellow eluate from the sugar column was evaporated to dryness under vacuum at 35°C, re-dissolved in iso-octane, and placed on a 2 × 15 cm silicic acid column previously washed with iso-octane. The carotenes contained in the sample are carried through the column with a solvent composed of 25 per cent chloroform and 75 per cent iso-octane, while the Q-255 compound remains near the top of the column. With 75 per cent chloroform and 25 per cent iso-octane, the Q-255 moves down the column. As a general practice, this band was never taken completely through the column but was moved only far enough away from other material at the top to permit a manual separation of the bands. After the portion containing the Q-255 was removed from the column, it was eluted with chloroform, reduced to dryness (under vacuum) and the dried material re-dissolved in absolute alcohol. The light absorption curves of such samples were determined between 210 and 300 mμ before and after the addition of a few grains of sodium borohydride.

For determining the amount of Q-255 in a sample, this abbreviated procedure proved to be of importance, since we observed a gradual decomposition of the compound the longer it was held on the column. The chromatographic technique used

for this separation is similar to that described by Crane and co-workers[10] for the Coenzyme Q from mitochondria. The following extinction coefficients were used to determine the amount of Q-255 in each sample: $E_{1\ cm}^{1\ per\ cent} = 212$, and $E_{1\ cm}^{1\ per\ cent}$ (oxidized-reduced) = 200. These values are in good agreement with those obtained for "Q_{254}" from alfalfa.[10] All spectral measurements reported in this paper were made either with a Beckman DU spectrophotometer or a Zeiss spectrophotometer, Model PMQ II.

Results.—The petroleum ether extracts from lyophilized chloroplasts, whether

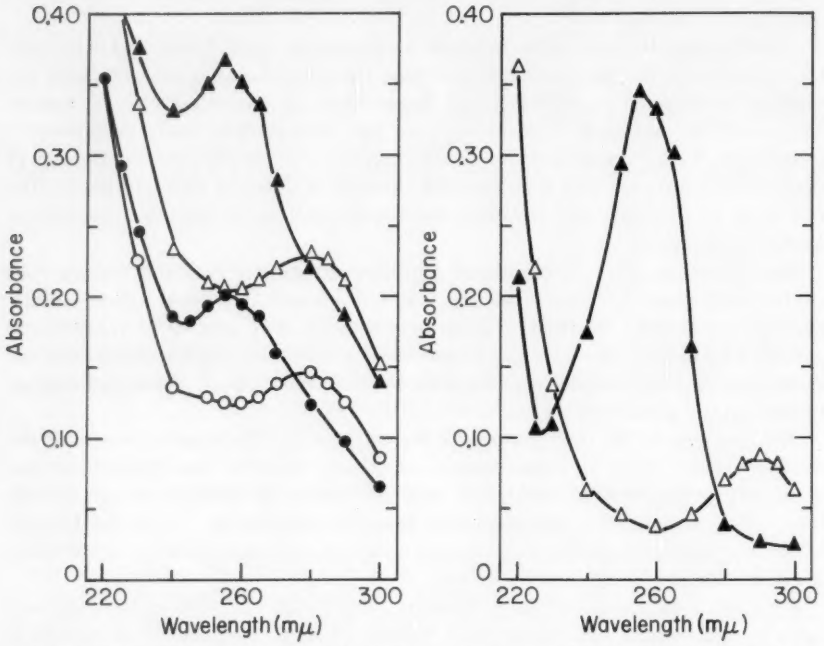

FIG. 1*a* (left).—Absorption curves of petroleum ether extracts (in absolute ethanol) of lyophilized sugar beet chloroplasts. Extract was divided into two equal parts, one chromatographed on a powdered sugar column(●—● = oxidized, ○—○ = reduced with sodium borohydride), and the other unchromatographed (▲—▲ = oxidized, △—△ = reduced).

FIG. 1*b* (right).—Absorption curve of purified Q-255 from sugar beet chloroplasts. ▲—▲ = oxidized, △—△ = reduced. 0.04 mg in 3 ml absolute ethanol.

those of sugar beet, spinach, or swiss chard, always contained Q-255. A typical example of such an observation is shown in Figure 1*a*.

For this curve, 100 mg of freeze-dried chloroplasts from sugar beet leaves were extracted with 100 ml of cold petroleum ether. The petroleum ether was split into two equal portions: One portion was evaporated to dryness and the residue re-suspended in absolute ethanol, while the other sample was chromatographed on a powdered sugar column as indicated in *Methods*. The curves showed that chromatographic treatment to remove the xanthophylls and carotenoids also decreases the amount of Q-255 present. This observation explains why after such chromatographic treatment the extract is not as active in restoring photochemical

activity as it was before such treatment. Previously, Lynch and French had experienced the same difficulty when magnesium oxide was used as the absorbent. In Figure 1*b* the absorption curves (oxidized and reduced) of purified Q-255 from sugar beet chloroplasts are shown.

The data in Table 1 give a comparison between the reactivating effects of equal molar concentrations of several different compounds. What is immediately appar-

TABLE 1

COMPARISON OF THE REACTIVATION OF THE FERRICYANIDE REDUCING CAPACITY OF EXTRACTED SUGAR BEET CHLOROPLASTS BY VARIOUS COMPOUNDS

Compound Added	Per cent of Initial Activity		
	a	b	c
Extracted-Control	56	26	35
.36 μM Q-255	116	97	109
.73 μM "	96	95	101
.36 μM Q-275	63	36	53
.73 μM "	42	31	47
.36 μM Vitamin K_3	47	52	53
.73 μM "	26	49	57
.36 μM β-Carotene	55	28	32
.73 μM "	52	26	34

Conditions: Temperature = 15°C; Gas phase = nitrogen; Red light. $[K_3Fe(CN)_6] = 5 \times 10^{-4}$ *M*, $[PO_4] = 0.05$ *M* (pH = 6.5), and [Chlorophyll] = 9.5×10^{-5} *M*. Each experiment (a, b, c) was performed on a new preparation of chloroplasts.

ent is the superior reactivating power of Q-255 not only over vitamin K but also over that of the Coenzyme Q compound isolated from mitochondria (Q-275), although structurally the two benzoquinones are quite similar. Menadione has only a slight reactivating effect at the concentration used; and, as reported earlier, purified β-carotene is without effect.

Because Lynch and French used dichlorophenol-indophenol as the oxidant in their experiments, and also since this dye has certain special qualities as a Hill reaction oxidant,[12] the reactivation of the photochemical activity with this dye as the Hill oxidant is compared in Table 2. These are the results of three different experiments,

TABLE 2

COMPARISON OF THE REACTIVATION OF THE DYE* REDUCING CAPACITY OF EXTRACTED SUGAR BEET CHLOROPLASTS BY VARIOUS COMPOUNDS

Compound Added	Per cent of Initial Activity		
	a	b	c
Extracted-Control	46	33	32
.36 μM Q-255	102	110	101
.72 μM "	79	93	98
.36 μM Q-275	61	37	40
.72 μM "	46	34	39
.36 μM Vitamin K_3	51	42	39
.72 μM "	56	46	43
.36 μM β-Carotene	43	33	31
.72 μM "	46	31	30

* Dichlorophenol-indophenol.
Conditions: Temperature = 15°C; Gas phase = nitrogen; Red light. Dichlorophenol-indophenol = 3.33×10^{-5} *M*, $[PO_4] = 0.05$ *M* (pH = 6.5); and Chlorophyll = 1.9×10^{-5} *M*.

and there is no great deviation from the pattern seen in Table 1 where potassium ferricyanide was used as the oxidant.

It is difficult to determine the exact amounts of Q-255 which will restore the original photochemical activity because of the inaccuracy inherent in the method for re-addition of the compound. The procedure, as outlined previously, entails an evaporation of a petroleum ether solution of the substance tested in the presence of the extracted chloroplasts. With such a method there is, a priori, as much chance that the compound will be left on the walls of the container as on the surface of the

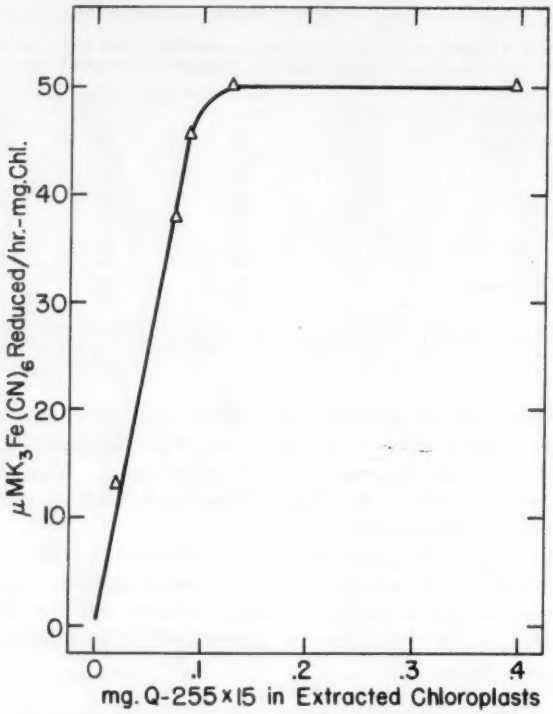

FIG. 2.—Curve showing the relation between Hill reaction activity and content of Q-255 retained in sugar beet chloroplasts after petroleum ether extraction. 100 mg lyophilized chloroplasts extracted with 10 ml, 20 ml, 30 ml, and 40 ml petroleum ether and the photochemical activity of 6.6 mg chloroplasts determined. 100 mg chloroplasts contained 4.11 mg chlorophyll and 0.40 mg Q-255. Temperature = 15°C. Light = saturating red. Gas phase = N_2. (See text.)

chloroplasts. Unfortunately, this direct evaporation is the only way found, so far, which will reactivate extracted chloroplasts.

It is possible, however, to determine the relationship between Q-255 concentration in the chloroplasts and photochemical activity by successively extracting the chloroplasts with petroleum ether and determining, after each washing, the photochemical activity and the amount of Q-255 removed from, or still retained in, the chloroplast. In Figure 2 the results of such an experiment are shown. For this particular experiment the first extraction does not alter the activity of the chloro-

plasts, although about 70 per cent of the Q-255 contained in the sample was removed. It is of interest, however, that the reactivity of the Hill reaction extrapolates to zero for complete extraction of the quinone. For this experiment the saturation rate was attained with only 30 per cent of the total content of the quinone. Presumably, this is due to the low activity of this preparation when compared to the intact chloroplast. Consequently, with chloroplast preparations with greater activity, the saturation rate would be attained at higher concentrations of the quinone.

From the data contained in Figure 2, it is possible to calculate the mole-ratio of Q-255 to chlorophyll. Such a calculation (using an average molecular weight of 906 for chlorophyll and 764 for Q-255) yielded a ratio of Q-255/chlorophyll = 0.12. Similar values were also obtained for spinach and swiss chard chloroplasts. This, we feel, is probably a minimal ratio since a portion of the Q-255 is lost during the extraction and purification procedures. We have extracted the same substituted *p*-benzoquinone from a blue-green alga, *Anacystis nidulans*, and from several green algae, including *Chlorella pyrenoidosa*, *Ankistrodesmus braunii*, *Chlamydomonas moewusii*, and *Scenedesmus obliquus*. In extracts of the photosynthetic bacterium *Rhodopseudomonas spheroides*, this compound was not observed. However, here we observed a reducible substance with an absorption maximum at 272 mμ. It is probably related to the quinone obtained from two other photosynthetic bacteria, *Rhodospirillum rubrum* and *Chromatium*.[13]

Discussion.—In 1937, Robert Hill succeeded in what other experimenters previously had attempted in vain; namely, to divide the process of photosynthesis into at least two characteristically different parts. The existence of dissimilar partial reactions had been deduced earlier from studies involving the entire process. Hill found that properly isolated chloroplasts in the presence of suitable substrates other than carbon dioxide could still evolve oxygen photochemically, while the artificial substrates themselves became reduced.[14] During the twenty-odd years since that discovery, much additional information has been gathered about this process, often called the "Hill reaction."[15, 16] Beyond the fact, however, that native chlorophyll is involved, only one other substance, manganese ion, has been shown to be essential for this process.[17, 18] Recently, as said above, Lynch and French discovered the reversible inactivation of chloroplast activity by petroleum ether extraction. This exciting observation amounts to a further subdivision of the system, since it demonstrates that a component essential for the release of oxygen can be reversibly removed.

When we re-examined this problem, we found that β-carotene, the first substance under suspicion,[1] was not the responsible entity. Griffiths *et al.*,[19] working with photosynthetic bacteria, found that in their organisms the carotenoids participate in a process unrelated in essence to that assumed by Lynch and French. They reported that carotenoids prevented the photooxidation of the chlorophyll, an effect previously observed with chlorophyll in organic solution.[15]

Our results obtained with vitamin K derivatives appeared to be the definitive answer, except that, as stated in this paper, no significant amount of vitamin K could be found in the chloroplast extracts. In its place we discovered comparatively large amounts of Q-255, a benzoquinone. What is the exact mode of action of this substance in the photosynthetic process? It would be of little practical value to

formulate an involved reaction mechanism involving this benzoquinone at the present time before we have studied, as we plan, its reactivity in various photochemical processes. It seems obvious that this substance is involved somewhere in the electron and hydrogen transport of the Hill reaction like Coenzyme Q_{10}, whose function in electron transport in mitochondrial systems has been clearly established. The question is where exactly it fits into the pattern of normal photosynthesis, i.e., in the transport of hydrogen or electrons preceding the reduction of carbon dioxide. The hypothesis that it may be a specific intermediate in the course of oxygen production cannot be decided on the basis of our experiments on the Hill reaction alone. Purple bacteria which do not evolve oxygen in the light also contain large amounts of such a benzoquinone. This, however, is again not sufficient to prove that Q-255 is not specifically involved in oxygen evolution since the bacterial quinone is different and is characterized by an absorption peak at 272 mμ. From a purely theoretical viewpoint, Q-255 should be capable of most of the reactions tentatively assigned to vitamin K in the photosynthetic process by Wessels.[15] The experiments reported here and the abundance of this compound in the chloroplasts of higher plants and in green algae make it extremely probable that it is a catalyst truly involved in the photosynthetic process. Furthermore, Lester and Crane[13] have shown recently that Q-255 is also contained in two representatives of marine algae: the red alga *Polysiphonia* sp. and the brown alga, *Fucus* sp. These observations strengthen the notion that this particular natural quinone is confined to photosynthetic tissue.

The suggestions and criticisms of Professor Hans Gaffron during the preparation of this manuscript are gratefully acknowledged.

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DETECTION, ASSAY, AND PRELIMINARY PURIFICATION OF THE PIGMENT CONTROLLING PHOTORESPONSIVE DEVELOPMENT OF PLANTS

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Responses of plant materials to radiation indicate that flowering and many other aspects of development are controlled by a reversible photoreaction[1,2] involving two forms of a pigment, with action maxima near 660 and 735 mμ. The photoreversible pigment can readily be changed from one to the other form, as indicated by response of the plant to irradiation in the region of the appropriate action maximum. Because the nature of the enzymatic action involved is still unknown, it appeared that direct observation of the pigment in the living material and an assay for its isolation would have to be based on spectrophotometric methods. The pigment should show a change in absorption at 655 and 735 mμ following conversion by radiation.

The location of the pigment and its concentration in specific cells are evidenced by the photoinduced formation of anthocyanin, which depends upon energy transfer from both forms of the excited pigment.[3] The concentration based on a molar absorptivity of 1×10^5 is estimated to be the order of 10^{-6} molar in the most effective cells and probably about 10^{-7} molar in the average tissue.[4] A spectrophotometer suitable for detecting this low concentration of the pigment in tissue must measure absorption of radiation with high sensitivity in dense light-scattering material. Such measurements cannot be made with commercial instruments.

Instrumentation.—The presence of the photoreversible pigment in intact tissue has been demonstrated with a recording, single-beam spectrophotometer. This spectrophotometer[5] employs an end-window multiplier-type phototube placed directly behind the sample to collect a large fraction of the transmitted light. The sample is illuminated by monochromatic light from the exit slit of a double, prism monochromator. Spectral measurements can be made on light-scattering samples having optical densities between 0 and 6, with a sensitivity as high as 0.1 for full scale deflection. The noise level is equivalent to an optical density change of 0.002 for samples having an optical density less than 4.

Since this is a single-beam instrument the recorded curve includes the spectral response of the instrument in addition to the absorption characteristics of the sample. The system response is sufficiently reproducible that valid difference spectra can be obtained by subtracting one recorded curve from another. When the spectral curve is very steep in the region of interest, an electrical compensation can be applied to alter the slope of the curve. This is achieved with a potentiometer geared to the wavelength drum which supplies an additional signal to the Y-axis. The compensation merely alters the system response to make it easier to compute difference spectra.

A more useful instrument for assay of the pigment is one which measures directly the optical density difference between two fixed wavelengths. Such an instrument,

which is a double beam, bichromatic spectrophotometer similar in principle to one developed by Chance,[6] is shown in Figure 1. The close juxtaposition of the end-window, multiplier-type phototube (P) and the sample allows dense light-scattering samples to be used. The two monochromatic beams are obtained with wedge interference filters F_1 and F_2. The spectral purity of the beams is improved with auxili-

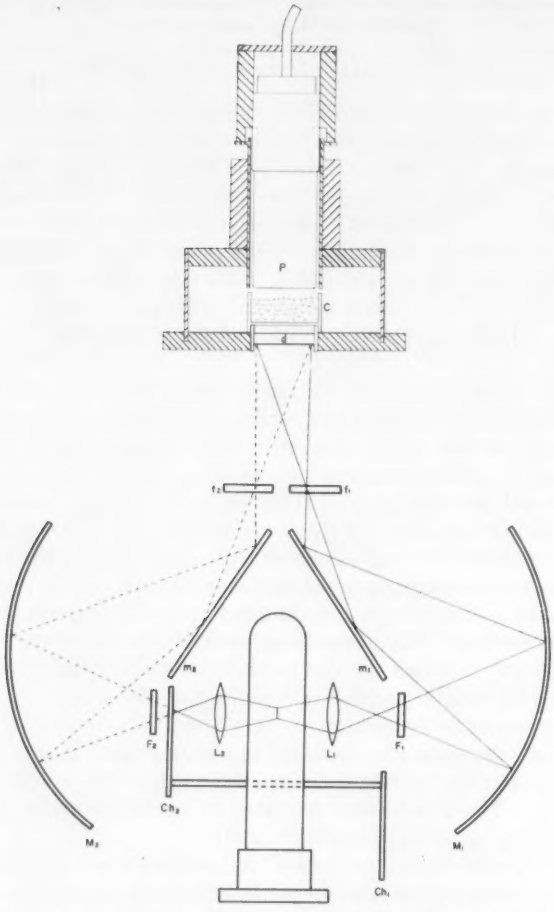

FIG. 1.—A differential spectrophotometer suitable for assay of the reversible pigment in living tissue or solution.

ary filters f_1 and f_2. The rotating chopper blades Ch_1 and Ch_2 cause the sample to be illuminated alternately with the two beams through the diffusing disk d. The phototube is synchronized with the chopper blades so that the measuring circuit records the optical-density difference ($O.D._{\lambda 1} - O.D._{\lambda 2}$). This instrument can be operated at an optical-density sensitivity as high as 0.05 for full-scale deflection, with a noise level of 0.0005.

The radiation source to change the state of the pigment was a focused beam from a 75 watt internal-reflection projection lamp with appropriate filters. The "red" source was a band in the region of 600 to 700 mμ and the "far-red" source in the region >700.

Detection of the Pigment in Living Plants.—The first tissue selected for examination was cotyledons of turnip seedlings (*Brassica rapa* var. white globe purple top) grown in the dark in the presence of chloramphenicol. This selection was based on the marked capacity of such tissue to form anthocyanin in light[7] and on the low content of protochlorophyll and chlorophyll which interfere with observations in the region of 670 mμ. The cotyledons were loosely pressed into the sample holder to a depth of about 1.5 cm and irradiated for about a minute with red radiation. This radiation converted any protochlorophyll present to chlorophyll and the pigment to the form with its absorption maximum near 735 mμ (later referred to as P_{735}). The curve of the optical densities of this tissue versus wavelength was measured in the 570 to 850 mμ region. The pigment was then converted to the form with the absorption maximum near 655 mμ (later referred to as P_{655}) by irradiation with far-red radiation for about a minute, and the optical density curve of the tissue was recorded again in the 570 and 850 mμ region.

The pigment was clearly evident by the optical density changes. Far-red radiation caused the optical density to decrease in the far-red region of the spectrum with a maximal change at 735 mμ and to increase in the red region with a maximal change at 655 mμ. Red radiation had the opposite effect.

A preliminary survey of etiolated parts of several seedling plants was then made and the shoots of 3-day-old maize seedlings were found to be particularly responsive. The shoots were cut and loosely pressed into a sample holder. A record from the spectrophotometer for a 1.5 cm thick sample of this material after irradiation with red and far-red radiation is shown in Figure 2. The difference spectrum is also plotted. It is evident that P_{735} has an absorption maximum at 735 mμ and P_{655} has an absorption maximum at 655 mμ. The reversibility between P_{655} and P_{735} by the action of red and far-red light can be repeatedly demonstrated. These results are in complete agreement with numerous action spectra[8] as also is the absorption in the region of 600 to 650 mμ.

The chlorophyll absorption near 672 mμ in Figure 2 is the result of the conversion of protochlorophyll by the initial irradiation with the red source. For a short period after the initial conversion, absorption in the red region of the spectrum changes because of a shift of the newly formed chlorophyll with an absorption maximum at about 680 mμ to a form with an absorption maximum at about 670 mμ.[9] Subsequent shifts of the chlorophyll peaks are sufficiently slow as not to interfere with the assay for the photoreversible pigment. No resynthesis of protochlorophyll is evident in maize seedlings for about 2 hours after the initial irradiation. Thereafter, the assay for the pigment is complicated by the snythesis of protochlorophyll and its conversion to chlorophyll by red irradiation.

A sample of maize shoots examined on the differential spectrophotometer gave a $\Delta(\Delta O.D.) = (O.D._{655} - O.D._{735})$ far-red irradiated $- (O.D._{655} - O.D._{735})$ red irradiated $= 0.2$. The concentration of the pigment and the sensitivity of the instrument were such that the distribution of the pigment along a single shoot could be determined by limiting the sample to a 5 mm segment. Values of $\Delta(\Delta O.D.)$

of 0.01 were observed with the coleoptile and 0.005 with the mesocotyl. Leaves of the maize seedlings were not responsive.

It was immediately evident from a preliminary survey of several tissues that the reversible pigment occurs at the maximum observed levels in shoots of several grasses irrespective of anthocyanin formation. Seemingly, the better guide is the responsiveness to etiolation of the shoots of gramineous plants which is displayed by the lengthening of internodes beneath the soil. Maize was selected as a suitable

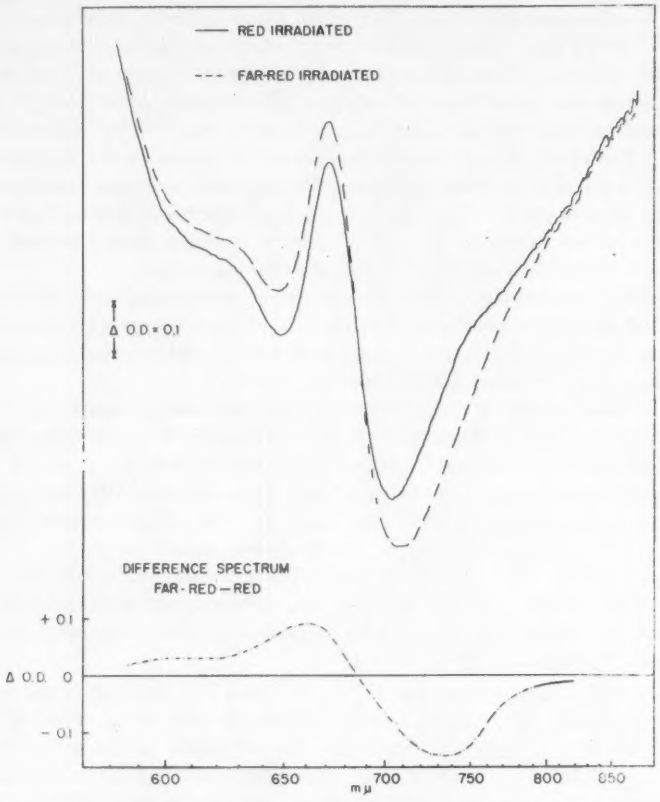

FIG. 2.—Recorded optical density curves from maize shoots in the 580 to 850 mμ region after red and far-red irradiations. The difference spectrum is shown.

source for the pigment because the seed are readily available, the shoots are large, and the pigment response is as great as with any tissue surveyed.

Separation of the Pigment.—Initial stages of pigment purification were effected by straightforward methods of protein chemistry using the Δ(ΔO.D.) values for assay.

A 450 gram lot of 3-day-old seedling maize shoots grown in darkness at 27° of variety US-13 was run in a blendor for 2 min with 450 ml of 0.1 *M* $Na_4P_2O_7$ buffer (pH 8.4) containing 0.01 *M* ascorbate and 0.01 *M* cystiene. The blended material

was passed through cheesecloth and the filtrate was then centrifuged at 40,000 × *g* for 20 min. The supernatant was centrifuged at 140,000 × *g* for 120 min and the resulting supernatant was brought to 0.33 saturation with $(NH_4)_2SO_4$. The precipitate was stirred for 15 min. It was then removed and redissolved in 0.25 of the original volume of solution. All operations were performed at 2°. Results from one of five runs are shown in Table 1. Protein values in this table were determined by the biuret method. The precision of the Δ(ΔO.D.) values was <0.0005/cm.

The pigment was retained upon dialysis at 2° against buffer at pH 8.4. The photoreversibility was retained for a period of at least two weeks upon holding solutions at −15°. It was lost upon heating to 50°.

A solution of the pigment gave the response shown in Figure 3 on the differential spectrophotometer. The reversibility of the pigment is also illustrated. In fact,

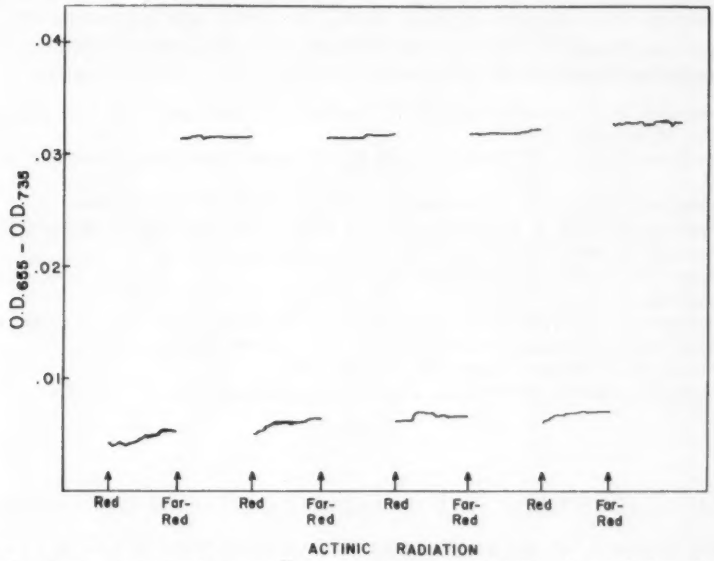

FIG. 3.—Photoreversible response to red and far-red radiation of a solution obtained from maize shoots.

TABLE 1

PURIFICATION FROM 450 GM. OF DARK-GROWN SHOOTS OF SEEDLING MAIZE FOR THE PIGMENT CONTROLLING PHOTORESPONSIVE DEVELOPMENT OF PLANTS

Fraction	Volume ml	Protein gm	Δ(ΔOD) /cm	Purification factor
40,000 × *g* supernatant	590	6.25	0.004	1.0*
140,000 × *g* supernatant	550	3.85	0.004	1.6
$(NH_4)_2(SO_4)$ 0.0 to 0.33 saturation	135	3.02	0.012	6.0

* Subject to some error from change in optical path-length by scattering.

it is necessary to lower the intensities of the analyzing light beams by about 100-fold, relative to the arrangement used for higher optical densities of unseparated plant material to prevent the beams from driving the reaction. This stage of purification brings the assay to the threshold of usual laboratory procedures. The presence of

the pigment in a clear solution of the 0.33 $(NH_4)_2SO_4$ saturated precipitate has been detected by means of $\Delta(\Delta O.D.)$ values obtained from the four appropriate measurements on conventional spectrophotometers.

Discussion.—Although many aspects of the nature of the pigment effective for control by light of plant development were found during the last seven years, attempts to separate it in several laboratories were unsuccessful and were usually left undescribed. This work supplies three needed elements for further progress: A source of the pigment, a method of assay, and a system for separation. There would seem to be no essential barrier to finding the nature of the enzymatic action of the pigment, P_{735}, which constitutes the limiting pacemaker[10] or "bottleneck" of control evident in plant development and to elaborating physiological and biochemical aspects of its action.

Summary.—The photoreversible pigment controlling many aspects of plant development was observed in living tissue by direct spectrophotometry. The pigment was separated from the tissue by usual methods of protein chemistry using differential spectrophotometry for assay.

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*MOLECULAR INTERACTION OF ISOALLOXAZINE DERIVATIVES. II**

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Understanding of the mode of action of the flavoproteins might be aided greatly by systematic study of the interaction of *iso*alloxazine derivatives with nonprotein compounds of known structure and state. The present paper describes spectrophotometric and potentiometric experiments which extend observations reported previously.[1]

Experimental.—Materials: 3-Methyllumiflavin (3,6,7,9-tetramethyl*iso*alloxazine) was synthesized by condensation of methylalloxan[2] with 2-amino-4,5-dimethyl-*N*-methylaniline, prepared by a modification of the method of Kuhn and Reinemund.[3] Melting point: 290°–291°C, with decomposition. Analysis: calculated, C 62.19, H 5.22, N 20.74; found, C 62.20, H 5.11, N 21.54. All other chemicals

were commercial preparations, purified as required. Solutions were freshly prepared immediately before use, and were carefully protected from light.

Methods: Absorption spectra were determined with the use of Cary model 11, Spectracord model 3000, and Zeiss model PMQ II spectrophotometers. Cuvettes were fitted with ground-glass stoppers, and varied in optical path length from 0.1 mm to 10 cm. In experiments requiring close control of temperature, jacketed cuvettes were used in conjunction with a circulating water bath. Determination of apparent dissociation constants was carried out as described previously.[1] Unless noted otherwise, measurements were made at 22.5° ± 0.5°C.

The apparatus and procedures used in oxidation-reduction potentiometry were essentially those described in detail in an earlier publication.[4]

Results.—Studies with different solvents: The absorption spectra of 3-methyllumiflavin in 24 solvents, listed in Table 1, were found to be of three major types in the

TABLE 1

SPECTROPHOTOMETRIC DATA FOR 3-METHYLLUMIFLAVIN IN DIFFERENT SOLVENTS

Solvent	Absorption maxima: Visible	Near-ultraviolet	Type of spectrum*
Anisole	476, 450	341	1*c*
Benzene	476, 450	339	1*c*
Benzyl alcohol	451	359	1*b*
Benzyl ethyl ether	476, 450	...	1*c*
Carbon tetrachloride	474, 447	335	1*c*
Chlorobenzene	478, 451	344	1*c*
Chloroform	474, 450	347	1*b*–1*c*
Chloronaphthalene	482, 455	...	1*c*
Cyclohexene	446	342	1*b*
Dioxane	442	334	1*b*
N,N-dimethylacetamide	448	338	1*b*
N,N-dimethylformamide	448	344	1*b*
Ethyl ether	470, 446	336	1*b*–1*c*
Formamide	448	362	1*a*–1*b*
Isoamyl alcohol	449	348	1*a*–1*b*
Mesitylene	476, 450	333	1*c*
Methanol	444	350	1*a*–1*b*
Phenol	454	370	1*a*
Phenyl ether	477, 449	340	1*c*
Piperidine	446	337	1*b*
Pyridine	450	343	1*b*
Tetrahydrofuran	445	337	1*b*
Tetralin	450	...	1*b*
Water	444	369	1*a*

* The designations 1*a*, 1*b*, and 1*c* refer to the correspondingly numbered figures, and indicate the spectrum most nearly resembled.

visible region, as illustrated in Figures 1*a*–1*c*. Also shown in Figures 1*a*–1*c* is the great variation among different solvents in the wavelength of maximum absorption in the near-ultraviolet. The position of the near-ultraviolet band appears to reflect the degree to which hydrogen bonding to *iso*alloxazine plays a role in solvent-flavin interaction. In systems where hydrogen-bond formation to *iso*alloxazine is precluded, the absorption maximum in this part of the spectrum ranges from 333 mμ to 344 mμ; in chloroform, a relatively poor hydrogen-bonding agent, the maximum is found at 347 mμ; in the alcohols studied, peak absorption occurs between 348 and 359 mμ; in water, the maximum is at 369 mμ. In anisole the near-ultraviolet band is at 341 mμ, whereas in phenol it is at 370 mμ. In dimethylacetamide maximal absorption occurs at 338 mμ, in formamide at 362 mμ. Addition of trichloro-

acetic acid to a solution of 3-methyllumiflavin in carbon tetrachloride shifts the band in the near-ultraviolet from 335 mμ to 350 mμ. Of possible interest is the fact that riboflavin in water at pH 7 has a band at 373 mμ, whereas at pH 12.5, where the predominant species is the anion, the band is at 355 mμ.

Compounds incapable of hydrogen-donor action, but which readily can be shown to interact with 3-methyllumiflavin in water solution, fail, at comparable con-

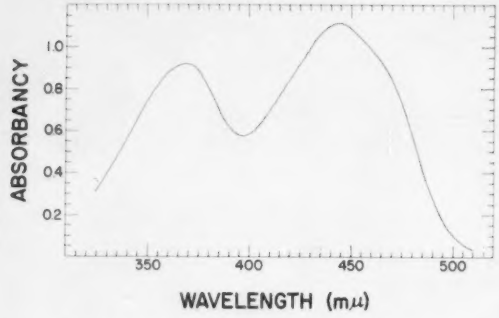


FIG. 1a.—Spectrum of 3-methyllumiflavin in water, pH 7.3; 1.9×10^{-5} *M* solution, 5 cm cuvette.

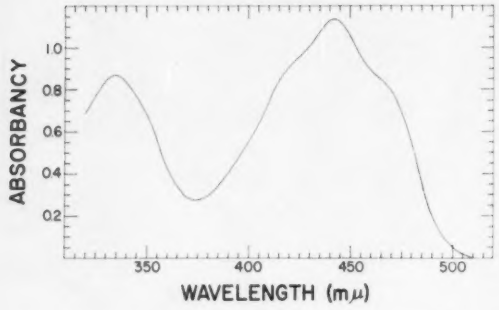


FIG. 1b.—Spectrum of 3-methyllumiflavin in dioxane; 1.9×10^{-5} *M* solution, 5 cm cuvette.

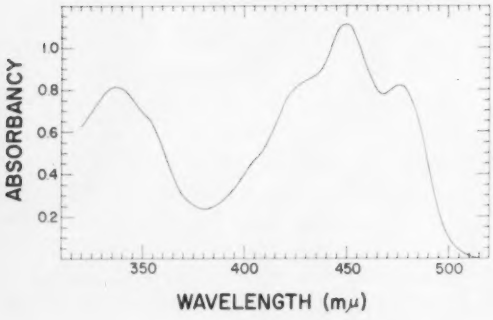


FIG. 1c.—Spectrum of 3-methyllumiflavin in benzene; 1.9×10^{-5} *M* solution, 5 cm cuvette.

centrations, to alter the visible and near-ultraviolet spectra of 3-methyllumiflavin in systems where all hydrogen bonding to *iso*alloxazine is excluded. For example, addition of 10^{-1} *M* caffeine to a 5×10^{-6} *M* solution of 3-methyllumiflavin in benzene is without effect on the spectrum recorded between 300 and 600 mμ. Di-

lution studies indicate it to be unlikely that this is ascribable to self-aggregation of the 3-methyllumiflavin or of the various interactants examined.

Most of the solvents are insufficiently miscible with water to permit examination of their effect on 3-methyllumiflavin in water solution. However, addition of pyridine to a solution of 3-methyllumiflavin in water reveals a weak but measurable interaction.[1] Weak interaction is observed also with benzoate; since acetate is without effect, it might be that benzene, if sufficiently soluble, would give comparable results. Naphthoate exhibits fairly strong interaction.[1]

Spectra recorded for 3-methyllumiflavin in water-pyridine mixtures, ranging from pure water to pure pyridine, do not display the form to be expected were there but two entities under observation. At certain wavelengths the absorbancy passes through a maximum or minimum as the mol fraction of pyridine is varied, and band maxima at certain compositions of mixed solvent are at wavelengths greater than those seen when 3-methyllumiflavin is dissolved in either solvent alone. The behavior is not unique to pyridine-water. Similar results are obtained, for example, upon examination of different mol ratios of benzene and phenol.

Compounds which cause no shift in spectrum upon addition to 3-methyllumiflavin in water solution, but which are potential hydrogen donors, do affect the spectrum observed in nonhydrogen-bonding solvents. Imidazole is without effect on the spectrum of 3-methyllumiflavin in water, but in benzene, even at the relatively low concentrations attainable, it causes a shift of the 339 mμ band to longer wavelengths and serves to lower the resolution in the visible region of the spectrum. Addition of methanol likewise alters both the visible and the near-ultraviolet parts of the spectrum. So also does addition of compounds like phenol, which in water function as what are believed to be charge-transfer interactants, but which, in solvents incapable of hydrogen bonding, emerge as hydrogen donors. Solvents such as dimethylacetamide, though not hydrogen donors, nevertheless preclude complex formation between 3-methyllumiflavin and added hydrogen donor, presumably by acting as competitive hydrogen acceptor.

3-Methyllumiflavin is insoluble in solvents such as *n*-heptane or cyclohexane, and carbon tetrachloride represents perhaps the nearest approximation to an "inert" solvent for this compound. Upon addition of 3-methyllumiflavin to carbon tetrachloride, enough appears to dissolve to permit spectrophotometric study in 10 cm cuvettes. The liquid is clear to the eye, displays no evidence of light scattering upon spectrophotometry, and shows no sign of separation upon ultracentrifugation at 60,000 $\times$ *g*. However, the flavin is readily retained on a sintered-glass filter or a short column of loosely packed glass wool. Solvents such as chloroform or dioxane easily elute flavin adsorbed in this manner, and addition to carbon tetrachloride of small amounts of such solvents prevents the adsorption from taking place. Evidently the forces holding 3-methyllumiflavin in solution in carbon tetrachloride are exceedingly weak. The low solubility in carbon tetrachloride has precluded the performance of adequate dilution tests, and as a result there is uncertainty about the state of aggregation of 3-methyllumiflavin in this solvent. This uncertainty seems unlikely, however, to invalidate deductions about gross relationships.

The possibility was tested that charge-transfer interaction with hydrogen-bonded *iso*alloxazine might be significantly favored over similar interaction with *iso*alloxazine free of hydrogen-bonding effects. The addition of α-chloronaphthalene to

3-methyllumiflavin in carbon tetrachloride leads to a measurable change in spectrum, and an apparent molar dissociation constant can be calculated for the system 3-methyllumiflavin: α-chloronaphthalene in carbon tetrachloride. The value obtained is ~3 M. To a 5×10^{-6} M solution of 3-methyllumiflavin in carbon tetrachloride, trichloroacetic acid was now added in a molarity approximately 20 times that of the flavin; this addition shifted the position of maximum absorption in the near-ultraviolet from 335 mμ to 350 mμ, and served also characteristically to diminish resolution in the visible range of the spectrum. The resulting solution was subjected to interaction with α-chloronaphthalene, and an apparent dissociation constant was determined in the usual way for the system 3-methyllumiflavin: α-chloronaphthalene, where the flavin now is in hydrogen-bonded form. A constant of 0.03 M was obtained. 3-Methyllumiflavin hydrogen bonded to trichloroacetic acid thus appears, in this system, to be a much more effective charge-transfer interactant than is nonhydrogen-bonded 3-methyllumiflavin.

Further studies with water solutions: Previous observations[1] on the interaction between flavin and L-tryptophan have been extended by measurement of the variation of K'_c with temperature. From the data shown in Figure 2, the value, $\Delta H = -6.1$ kcal./mole may be calculated for the process, riboflavin + L-trypotophan → riboflavin: L-tryptophan, examined in unbuffered solution, pH 6.9. Similar measurements were made for the interaction between riboflavin and the anion of pentachlorophenol.[5] At pH 9.2 and in unbuffered solution, the following values obtain for the process, riboflavin + pentachlorophenolate → riboflavin: pentachlorophenolate: ΔH, −3.3 kcal./mole; K'_c (dissociation) at 15° C, 0.0022 M.

Oxidation-reduction potentiometry has been used to compare the interactive effects displayed by oxidized flavin and reduced flavin, respectively. First results are summarized in Table 2. Failure of a compound to affect the potential observed

TABLE 2

ΔE_m VALUES

Interactant	Concentration M	pH	ΔEm* millivolts
Pentachlorophenol	0.05	8.10	−21
L-Tryptophan	0.025	6.61	−15
Serotonin†	0.025	4.50	−19
L-Histidine	0.20	6.65	0
Adenosine-5′-phosphate	0.05	6.15	0
Guanosine-2′(or 3′)-phosphate	0.05	6.18	0
Caffeine	0.08	6.18	+14
3-Hydroxy-2-naphthoic acid	0.37	6.73	−42

* Em ($FMN/FMNH_2$ + interactant) − Em ($FMN/FMNH_2$); 30°C.
† Serotonin-creatinine sulphate complex; creatinine at a concentration of 0.2 M is without effect on Em.

for the half reduced system (i.e., $\Delta E_m = 0$) indicates either an absence of detectable interaction or else equivalence of interaction with FMN[6] and $FMNH_2$. Since histidine is without effect on the visible and near-ultraviolet spectra of FMN in water, it is possible that for this compound a ΔE_m of zero indicates "no interaction." Purines, on the other hand, do display spectroscopic evidence of interaction with the oxidized forms of *iso*alloxazine derivatives,[7] and thus the results for adenylic and guanylic acids suggest equal interaction with oxidant and reductant. The data for adenylic acid are consistent with the observation[8] that, over the range of pH examined, the oxidation-reduction potentials of FAD are the same as those of FMN.

The values of $\Delta E_m \neq 0$ are small but measurable. Figure 3 illustrates the change in potential observed at varied concentrations of 3-hydroxy-2-naphthoate. Assumption of a 1:1 stoichiometry and application of the relationship

$$\Delta E_m = \frac{RT}{nF} \ln \frac{K'_o}{K'_r}$$

leads to the conclusion that K'_r, the apparent dissociation constant of $FMNH_2$:3-hydroxy-2-naphthoate, is some 25 times greater than K'_o, the apparent dissociation constant of FMN:3-hydroxy-2-naphthoate.

Discussion.—In a previous publication,[1] data were presented on the interaction in water solution between *iso*alloxazine derivatives (FMN, riboflavin, 3-methylribo-

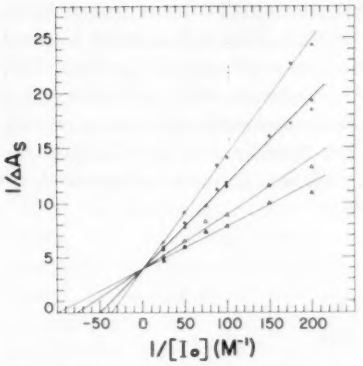


FIG. 2—Riboflavin: L-tryptophan system; water solution, unbuffered, pH 6.9; concentration of riboflavin, 1.9×10^{-5} *M*; jacketed 5 cm cuvette; open circles, 37.2°C; solid circles, 28.0°C; open triangles, 15.0°C; solid triangles, 8.0°C.

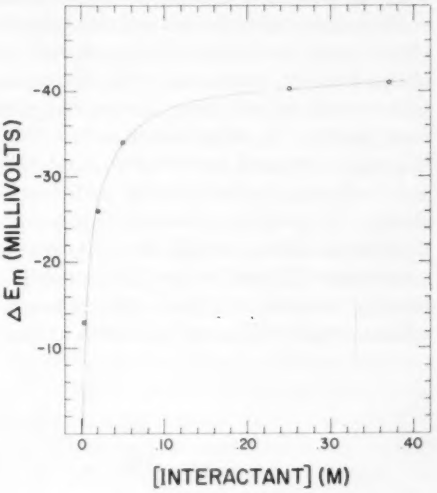


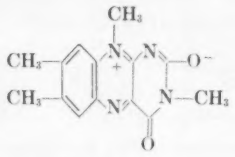
FIG. 3.—Change in oxidation-reduction potential as a function of interactant concentration; 30°C, pH 6.7; concentration of flavin mononucleotide, 4×10^{-4} *M*; interactant, 3-hydroxy-2-naphthoate; $\Delta E_m = E_m$ (FMN/$FMNH_2$ + interactant) − E_m (FMN/$FMNH_2$).

flavin) and various conjugated molecules. The interactants examined included representatives of the aromatic amino acids and purines, as well as compounds structurally related to certain vitamins, hormones, and agents known to uncouple electron transport from oxidative phosphorylation. Hydrogen bonding between interactant and *iso*alloxazine was found not to be a primary feature of interaction in the systems examined, and the suggestion was advanced that molecular charge transfer[9, 10] may be a more important factor. The experimental results reported for the interaction of *iso*alloxazine derivatives with L-tryptophan have since been confirmed by Isenberg and Szent-Györgyi.[11] However, the conclusion by these authors that the effect under study represents the formation of semiquinone differs importantly from the interpretation we have given, and, in our opinion, is in error.

The suggestion that the interactive effects observed in water solution might involve molecular charge transfer is supported by the calculations subsequently re-

ported by Pullman and Pullman[12, 13] for the ionization potentials and electron affinities of riboflavin and several of the interactants included in our first studies. Indeed, a number of papers have recently appeared in which interactive effects in systems containing riboflavin, pteridines, or diphosphopyridine nucleotide are ascribed to charge-transfer complex formation.[12–15] It should be emphasized, however, that neither our data nor those of others provide what can at this time be considered unequivocal evidence in favor of a charge-transfer interpretation; such an interpretation may appear plausible, but it remains to be proved.

Interaction between 3-methyllumiflavin and compounds incapable of action as hydrogen donor, though readily demonstrable in water solution, is, at similar concentrations, not observable in the visible and near-ultraviolet regions of the spectrum when all hydrogen bonding to *iso*alloxazine is excluded. The results of dilution studies suggest that the basis of this behavior lies elsewhere than in self-aggregation effects, and, since many of the solvents examined may be expected to engage in charge-transfer interaction with *iso*alloxazine, the interpretation presents itself that in such solvents there occurs competition with the added interactant for the *iso*alloxazine. In other instances, however, this interpretation seems less probable; it appears awkward, for instance, in relation to certain of the nonaromatic solvents, and the question arises whether additional factors may not also be involved. The changes in spectrum observed in going from hydrogen-bonding solvents to non-hydrogen-bonding solvents are reminiscent of observations with dyes of the merocyanine type,[16, 17] and the possibility merits serious consideration that, in hydrogen-bonding solvents, charge-transfer interaction with *iso*alloxazine is favored by an enhanced contribution of structures of the type:



This formulation appears consistent with the fact that caffeine can be shown to interact with 3-methyllumiflavin in water, in methanol and in formamide, but not in solvents unable to act as hydrogen-donor. It is consistent also with the observation that addition of α-chloronaphthalene to a carbon tetrachloride solution of 3-methyllumiflavin leads to an interactive effect with an apparent molar dissociation constant of $\sim 3\ M$, whereas addition of α-chloronaphthalene to a similar solution containing also approximately $10^{-4}\ M$ trichloroacetic acid gives an apparent dissociation constant of $0.03\ M$.

The potentiometric data show that the interactive effects under study are not limited to the oxidized form of *iso*alloxazine, but extend also to the reduced form. For example, the data given in Figure 3 for the system FMN/$FMNH_2$:3-hydroxy-2-naphthoate indicate a ratio of approximately 25 for the apparent dissociation constants of $FMNH_2$:3-hydroxy-2-naphthoate and FMN:3-hydroxy-2-naphthoate. In the case of caffeine, the reduced system displays the smaller apparent dissociation constant: addition of caffeine to FMN/$FMNH_2$ leads to a positive shift in potential. *Iso*alloxazine may serve[13] as electron acceptor in the oxidized systems

examined, and as donor in the reduced systems. The possibility suggests itself that charge-transfer complexes may act as intermediates in electron transfer between *iso*alloxazine and such other biologically important oxidation-reduction systems as, for example, the pyridine nucleotides and the vitamins K.

Grabe[18] recently has discussed a mechanism for oxidative phosphorylation at the DPN-FMN level, involving the formation of intermediates in which phosphate is linked to *iso*alloxazine and nicotinamide lying in parallel planes, as would be the case in a charge-transfer complex. If this hypothesis proves tenable, then the data reported above raise the question whether agents known to uncouple oxidative phosphorylation from electron transport, such as pentachlorophenol, might not act in part through competitive charge-transfer interaction with the *iso*alloxazine (and perhaps also the nicotinamide) component of such a complex.

Weber[7] has shown that with FAD in water solution, internal complex formation occurs between the *iso*alloxazine and adenine components of the molecule, and a comparison of the apparent dissociation constants of the systems riboflavin:adenine, riboflavin:caffeine, and 3-methylriboflavin:caffeine indicates[1] that in this complex formation molecular charge transfer may be a dominant factor. It may in time prove of interest that, although known to interact with *iso*alloxazine, adenosine-5′-phosphate is found to be without effect on the oxidation-reduction potential of $FMN/FMNH_2$. This result is consistent with the finding of Lowe and Clark[8] that the oxidation-reduction potentials of FMN and FAD are indistinguishable at all values of pH investigated, from pH 2.4 to pH 10.9, and indicates equality of the dissociation constants of FMN:AMP and $FMNH_2$:AMP. Of the interactants thus far examined, only guanylic acid (mixture of 2′ and 3′ phosphates) gives similar results. The dissociation constants of the few other *iso*alloxazine:interactant systems which have been tested all display dependence on the state of oxidation of the system. It may of course well be that the apparently special position of adenylic and guanylic acids is entirely fortuitous, and that further experiments will lead to the identification of a variety of compounds which interact equally with oxidized and reduced *iso*alloxazine. On the other hand, the structural requirements for the condition $K'_o = K'_r$ may sharply limit the possibilities, and it may be that adenylic and guanylic acids are indeed representatives of a rather select group of compounds, especially so if attention is restricted to substances of biological interest. Considerations such as this direct attention to the as yet unknown function of the purine component of FAD (and, similarly, the pyridine nucleotides), and lead to inquires about the role of *iso*alloxazine-purine interaction in the chemistry of flavoproteins.

Kuhn and his co-workers[19, 20] suggested that, in the "old yellow enzyme," bond formation occurs between the protein and the imino group in position number 3 of the *iso*alloxazine ring. The bond in question subsequently has come to be thought of as a hydrogen bond, most recently as a hydrogen bond involving the OH group of a tyrosine residue,[21, 22] and in this form Kuhn's early suggestion remains today a widely accepted formulation of *iso*alloxazine-protein interaction in the "old yellow enzyme" and, it often is assumed, in flavoproteins in general. The evidence in support of this almost certainly oversimplified formulation is, however, fragmentary and open to alternative interpretation.[1] The subject merits renewed experimental attention, and the results of the present studies may serve to indicate some of the

factors to be taken into consideration. Thus, the possibility of molecular charge transfer between *iso*alloxazine and aromatic amino acid residues of the protein would seem to deserve careful evaluation. The fact that in water solution interaction of *iso*alloxazine with L-tryptophan is favored over that with L-tyrosine or L-phenylalanine[1] of course directs particular attention to the tryptophan residues of flavoproteins. One would be remiss, however, not to explore also the possible role of tyrosine and phenylalanine residues; contrary to the report by Isenberg and Szent-Györgyi[11] that interaction of flavin with tyrosine or phenylalanine does not occur, such interaction has been demonstrated.[1] On the other hand, we thus far have found no evidence for charge transfer interaction in water solution between *iso*alloxazine and imidazole, although Pullman and Pullman in their recent paper[12] attribute to imidazole donor properties almost as good as those of tryptophan and better than those of tyrosine or phenylalanine.

An apparent correlation has been noted between the occurrence of hydrogen bonding and the wavelength of maximum absorption by protein-free flavin in the near-ultraviolet. Examination of the spectra reported for water solutions of some of the more highly purified flavoproteins indicates that here the near-ultraviolet band is located most frequently in the region 370–380 mμ, but varies in position over most of the range of wavelengths observed with nonprotein systems. If the attempt is made to apply to the flavoproteins the apparent correlation noted for non-protein systems, then the conclusion would follow that, even in water solution, there is considerable variation in the degree to which the *iso*alloxazine of flavoproteins is hydrogen-bonded. An interesting comparison is afforded by two crystalline FMN-proteins for which spectroscopic data are available both for the conjugated protein and for the protein free of FMN. In the case of glycolic acid oxidase of spinach,[23] the spectrum of protein-bound FMN has a band maximum at 338 mμ, and it thus might be that in this enzyme the *iso*alloxazine is predominantly in non-hydrogen-bonded form. In the "old yellow enzyme" from yeast,[24] on the other hand, the spectrum of protein-bound FMN resembles that of hydrogen-bonded *iso*alloxazine:non-protein systems, the near-ultraviolet maximum now occurring at 380 mμ, and so perhaps the *iso*alloxazine ring of the "old yellow enzyme" is subject to hydrogen-bond formation; whether to solvent or to groups of the protein is, of course, a moot question.

The interaction between *iso*alloxazine and protein in any one enzyme represents the resultant of a variety of effects, of which hydrogen bonding and charge transfer are but two, and this interaction will differ in important detail from one flavoprotein to another. Indeed, it will differ between the various states and compounds of a single flavoprotein, and a more basic understanding of the chemistry of flavoproteins in considerable measure awaits information about pertinent linked functions. The identification of protein groups to which flavin may be bound, and the recognition of the forms of interaction involved in such binding, constitute first steps only.

Summary.—Molecular interaction of *iso*alloxazine derivatives has been examined in a variety of solvent systems. Two effects have been encountered: hydrogen-bonding and interaction thought to have its basis in molecular charge transfer. Interaction with compounds which are charge-transfer donors but not hydrogen donors is increased greatly when the flavin is hydrogen-bonded to a solvent like water or to a solute such as trichloroacetic acid.

Previous studies of charge-transfer interaction in water solution have been extended to include data for reduced flavin mononucleotide, and values of ΔH have been determined for the interaction of riboflavin with L-tryptophan and with pentachlorophenolate. The results are discussed in relation to the chemistry of flavoproteins.

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† Predoctoral Fellow of the National Cancer Institute, United States Public Health Service.

‡ Predoctoral Fellow of the National Heart Institute, United States Public Health Service.

§ Summer Research Fellow of the United States Public Health Service, 1957; Summer Research Fellow of the National Science Foundation, 1958.

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[5] We are grateful to Dr. E. C. Weinbach for the gift of a highly purified sample.

[6] The following abbreviations are used: FMN, flavin mononucleotide; $FMNH_2$, reduced flavin mononucleotide; FAD, flavin adenine dinucleotide; AMP, adenosine-5′-phosphate; ΔE_m, the difference in potential between half-reduced oxidation-reduction systems.

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*ON BIOCHEMICAL EVOLUTION: LYSINE FORMATION IN HIGHER PLANTS**

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Two different pathways of lysine synthesis have thus far been found in the biological world: one, via α-aminoadipic acid, was first shown in the fungus, *Neurospora crassa*,[1, 2] and the other, via α,ϵ-diaminopimelic acid, in the bacterium, *Escherichia coli*[3] (cf. Vogel and Bonner[4]). Recent isotope studies have indicated that the route via diaminopimelic acid exists in all representatives of the "true bacteria"

and actinomycetes tested[5] as well as in green algae,[5, 6] but that the route via α-aminoadipic acid occurs in all the ascomycetous and basidiomycetous fungi investigated,[5–7] and also in a euglenid.[6] The consistency of these results has encouraged the view that certain biosynthetic pathways, as "biochemical organelles" representing appreciable periods of evolution, are characters of particular phylogenetic significance.[6] Accordingly, it seemed of interest to extend the comparative study of lysine synthesis to some higher plant materials. In the present investigation, two whole plants and four plant tissues have been examined with the aid of tracer methods similar to those which proved valuable in the analysis of microbial lysine formation.

Materials and Methods.—Organisms and tissues: The whole organisms used were the fern, *Azolla caroliana*,[8] and the duckweed, *Lemna minor*.[9] The tissues employed were (a) pollen tissue from *Ginkgo biloba* (maidenhair tree);[10] (b) leaf parenchyma tissue from *Agave toumeyana* (century plant);[11] (c) habituated root tissue from *Melilotus officinalis* (sweet clover);[12] and (d) petiole crown gall tissue from *Helianthus annuus* (sunflower).[13]

Media: Chemically defined media solidified with 1.5 per cent agar were used. For *Azolla* and *Lemna* and for the tissues derived from *Melilotus* and *Helianthus*, the medium was that of Nickell.[8] For the remaining two tissues, the basal medium was that of Tulecke,[14] modified by the omission of glycine and by the substitution of ferrous sulfate, in equimolar amount, for ferric citrate. In the case of the *Ginkgo* tissue, this modified basal medium was supplemented with 0.5 mg L-arginine hydrochloride and 0.05 mg ammonium sulfate per ml, and in the case of the *Agave* tissue, with 0.1 mg L-arginine hydrochloride and 0.05 mg ammonium sulfate per ml.[14] Radioactive substances were incorporated into the media, as indicated.

Cultivation: Aseptic cultures of the plant materials were grown in 20 ml. of solid medium in 6-inch screw-capped tubes (diameter, 1 inch). Each tube was inoculated with approximately 100 mg (wet weight) of the desired plant or tissue. *Azolla* and *Lemna* were incubated at 23° with illumination, and the tissues at 28° in the dark. The incubation period was about four weeks in all cases. During this period, the mass (wet weight) of the various plants and tissues increased some two to tenfold over the inocula employed.

Tracer methods: The general technique used consisted of measuring the relative specific radioactivity of the lysine and certain other amino acids obtained from the protein of each of the cultures, following growth of the latter in the presence of selected C^{14}-labeled compounds. For the determination of the relative molar abundance of these protein amino acids, uniformly labeled sucrose-C^{14} (0.25 microcurie per mg, Tracerlab, Inc.) was substituted for the unlabeled sucrose otherwise employed in the media.[2, 15] In all other experiments, radiochemicals were added to the media, as indicated; approximately 20 to 50 microcuries of C^{14} were used per culture tube. DL-Aspartic-3-C^{14} acid (Research Specialties Co.) and DL-aspartic-4-C^{14} acid (New England Nuclear Corp.) were employed at a concentration of 0.15 mg per ml and were brought (with sodium hydroxide) to the usual pH values of the media. Sodium acetate-2-C^{14} and DL-alanine-1-C^{14} (both from New England Nuclear Corp.) were used at concentrations of 0.05 and 0.1 mg per ml, respectively. At the end of the incubation period, the whole plants or the tissues obtained were harvested, dried *in vacuo*, ground in a mortar, and subjected to the extraction, hy-

drolysis, paper chromatography, and counting procedures previously described.[2, 15] The relative specific radioactivity of the desired protein amino acids was then computed, essentially as before,[2, 15] on the basis of their relative molar abundance.

Results.—The 3- and 4-labeled aspartic acids, acetate-2-C^{14}, and alanine-1-C^{14} used in the present study were selected because of their diagnostic value in analyses of lysine synthesis (cf. Vogel[6]). The results obtained with the higher plant materials are summarized in Table 1; analogous results with *Candida utilis* (ATCC No. 9950), as a representative of the higher fungi, are included for comparison. It is apparent that the labeling patterns of the intact higher plants (including a fern and a flowering plant) and of the tissues (including pollen, leaf, root, and petiole tissues derived, respectively, from a gymnosperm, a monocotyledon, and two dicotyledons) are very similar to one another. Thus, in all the higher plant materials, lysine, aspartic acid, and threonine exhibit approximately the same relative specific activity with either the 3- or the 4-labeled aspartic acid, or with the acetate-2-C^{14};[16] with alanine-1-C^{14}, the specific activity of the lysine is comparatively high.[17] These labeling patterns closely resemble those of bacteria and green algae,[5-7] but contrast sharply with that of *C. utilis*. As shown in Table 1, in the case of the latter organism, lysine is not appreciably labeled witn either aspartic-4-C^{14} acid or alanine-1-C^{14}, whereas acetate-2-C^{14} results in relatively heavy labeling.[17]

TABLE 1

INCORPORATION OF TRACERS INTO PROTEIN AMINO ACIDS
(AS SPECIFIC ACTIVITY RELATIVE TO THE RESPECTIVE PROTEIN ASPARTIC ACID VALUES)

Protein Amino Acid	*Azolla*	*Lemna*	*Ginkgo* (pollen tissue)	*Agave* (leaf tissue)	*Melilotus* (root tissue)	*Helianthus* (petiole tissue)	*Candida*
DL-Aspartic-3-C^{14} Acid							
Aspartic acid	100	100	100	100	100	100	100
Threonine	96	63	109	75	100	90	116
Lysine	92	75	100	76	129	105	56
DL-Aspartic-4-C^{14} Acid							
Aspartic acid	100	100	100	100	100	100	100
Threonine	80	60	102	80	109	86	111
Lysine	91	62	71	88	118	86	<5
Sodium Acetate-2-C^{14}							
Aspartic acid	100	100	100	100	100	100	100
Threonine	81	87	108	82	107	107	109
Lysine	101	108	115	95	116	105	448
DL-Alanine-1-C^{14}							
Aspartic acid	100	100	100	100	100	100	100
Threonine	83	68	119	115	122	75	88
Lysine	223	147	805	410	648	322	<5

Discussion.—As recently reviewed,[18] a number of earlier investigations of amino acid metabolism in higher plant materials have been conducted with the aid of uniformly labeled aspartic acid as well as of labeled acetate and alanine. Certain of these investigations are relevant to the lysine-threonine-aspartic acid interrelalation, although details of lysine synthesis apparently have thus far not been revealed in higher plants. Bilinski and McConnell found that acetate-1-C^{14} or acetate-2-C^{14} is incorporated into various amino acids in wheat gluten, among them, lysine, aspartic acid, and threonine.[19] On the basis of degradation studies, these authors were able to show that aspartic acid provides the carbon skeleton for threo-

nine; a similar conclusion was reached by Naylor *et al.* from experiments with C^{14}-aspartic acid as tracer.[20] The relatively low specific activity of lysine observed by Bilinski and McConnell (in contrast to the present findings) seems to indicate that in their experiments the flow of labeled metabolites was rather remote from a steady-state condition.[21] Fowden and Bryant reported labeling of lysine after absorption of C^{14}-aspartic acid by roots of *Convallaria* or *Polygonatum*;[22] however, since C^{14} from randomly labeled aspartic acid can enter lysine via either of the two known types of lysine path (cf. Vogel[6]), their findings did not make it possible to determine which type of path functions in these plants.

The present experiments indicate that, in higher plants, (*a*) aspartic acid (with equivalent utilization of its 3- and 4-carbon atoms) is a precursor of lysine as well as of threonine; (*b*) acetate makes essentially equivalent contributions to aspartic acid, lysine, and threonine; and (*c*) the 1-carbon of alanine (presumably via that of pyruvate) is incorporated more heavily into lysine than can be accounted for by the conversion of the alanine to aspartic acid. Although alternative interpretations have not been excluded, these results suggest that the mechanism of lysine synthesis in higher plants resembles that in the bacteria and the green algae studied[5–7] and may involve α,ϵ-diaminopimelic acid as an intermediate.

Summary.—With DL-aspartic-3-C^{14} acid, DL-aspartic-4-C^{14} acid, acetate-2-C^{14}, and DL-alanine-1-C^{14} as tracers, lysine synthesis has been studied in a whole fern (*Azolla*), a whole flowering plant (*Lemna*), and in pollen, leaf, root, and petiole tissue cultures derived, respectively, from a gymnosperm (*Ginkgo*), a monocotyledon (*Agave*), and two dicotyledons (*Melilotus*, *Helianthus*). All these plant materials showed lysine-threonine-aspartic acid labeling patterns like those of the bacteria and the green algae previously examined, but unlike those of higher fungi.

The author is very greatly indebted to Dr. L. G. Nickell and Dr. W. Tulecke for their generosity in making available all the plants and tissues used in this study and in supplying much valuable information. Stimulating discussions with Dr. D. M. Bonner and Dr. K. V. Thimann are also gratefully acknowledged. Excellent technical assistance has been provided by Mrs. Helen Carnevale.

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[16] The rather low values for threonine and lysine obtained with both labeled aspartic acids in *Lemna* probably are significant and may indicate a "channeling" of aspartic acid, i.e., a preferential utilization of aspartic acid for incorporation into protein rather than for conversion to threonine or lysine. It will be noted, though, that the labeling of the lysine and the threonine are approximately equal, and that with acetate-2-C^{14}, all three protein amino acids exhibit nearly the same specific activity.

[17] Probable mechanisms underlying the various labeling patterns observed will be discussed elsewhere.

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[21] Absence of such a steady-state condition may also account for the finding of P. Linko (*Acta chem. scand.*, **12**, 101 (1958)) that aspartic acid was the only labeled amino acid in the protein of excised leaves of *Convallaria*, following their exposure to C^{14}-aspartic acid.

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*INHIBITION BY PUROMYCIN OF AMINO ACID INCORPORATION INTO PROTEIN**

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Puromycin[1] is an antibiotic produced by the actinomycete, *Streptomyces albo-niger*.[2] It has a broad range of biological activity, being effective against bacteria, protozoa, parasitic worms,[3] an alga,[4] and cells of mammalian origin.[3, 5] The site of its action has remained obscure although the compound was shown to be an inhibitor of induced enzyme synthesis.[6]

The structure of puromycin has been determined[7] and confirmed by total synthesis.[8] As can be seen from Fig. 1*a*, the antibiotic consists of an aminonucleoside linked to an amino acid. The close structural resemblance between puromycin and the amino acid-bearing end of soluble (or more exactly transfer) ribonucleic acid (S-RNA)[9, 10, 11] is shown in Figure 1. Although the bond between the amino acid and the remainder of the molecule differs in the two compounds, the peptide bond does bear a resemblance to the ester bond. For example, enzymes which catalyze the hydrolysis of one will often catalyze the hydrolysis of the other.[12] The considerations mentioned above suggested to us that the antibiotic action of puromycin is due to an inhibition of protein synthesis, specifically at a site involving transfer RNA.

At least three steps in protein synthesis may be readily distinguished owing to the efforts of a larger number of workers than can be cited here. These steps may be written:[13]

(1) $AA + ATP + E \rightleftarrows E\text{-}AMP\text{-}AA + PP$

(2) $E\text{-}AMP\text{-}AA + S\text{-}RNA \rightleftarrows S\text{-}RNA\text{-}AA + AMP + E$

(3) $S\text{-}RNA\text{-}AA + Ms \xrightarrow[ATP]{GTP} Ms\ protein + S\text{-}RNA.$

The purpose of this paper is to demonstrate that puromycin, at the low levels required for biological activity, inhibits the incorporation of radioactive amino acid into protein and further to specify its site of action by an examination of the separate steps enumerated above. Our experiments have been confined to a study of the effects of puromycin upon activation and incorporation of leucine in extracts of rat liver.

Materials and Methods.—Puromycin hydrochloride (Lot #1315B-179) was kindly supplied by Dr. E. C. De Renzo of Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York. Radioactive leucine was either DL-leucine-1-C^{14}, 42 μC per mg, Nuclear-Chicago Company, and a gift of Dr. A. T.

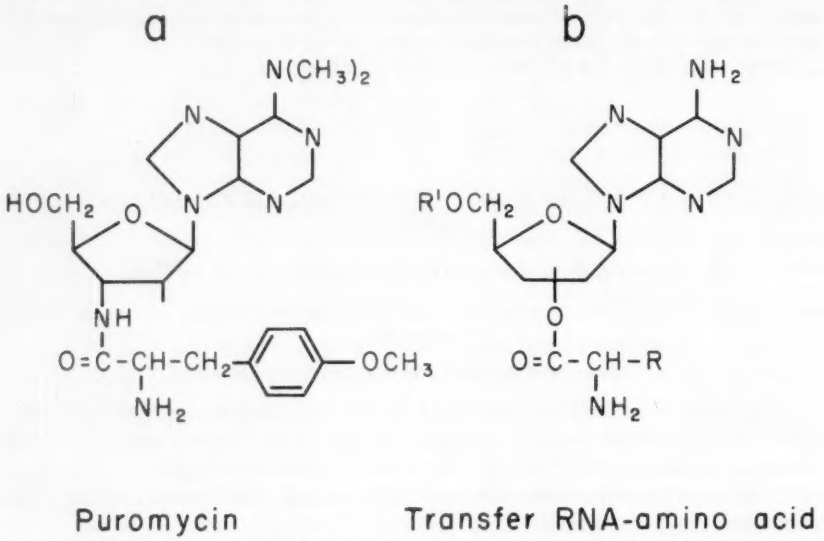

FIG. 1.—Structures of puromycin and the amino acid-bearing end of transfer RNA. R represents the remainder of the amino acid residue, R′ represents the remainder of the RNA polymer.

Jagendorf, or L-leucine-U-C^{14}, 50 μC per mg, purchased from the Schwarz Laboratories. L-leucine and the sodium salt of GDP were purchased from the California Corporation for Biochemical Research. The sodium salt of GTP and the crystalline sodium salt of ATP were obtained from the Pabst Laboratories. The barium salt of phosphoglyceric acid, Schwarz Laboratories, was converted to the sodium salt before use. The sodium salt of phosphocreatine was puchased from the Sigma Chemical Company. Pyrophosphate labeled with P^{32}, prepared by pyrolysis of orthophosphate-P^{32}, was a gift of Dr. M. Bessman. Dr. G. L. Cantoni generously furnished ATP-creatine transphosphorylase, 2.1 mg protein per ml, prepared by the method of Noda *et al.*,[14] and S-RNA-L-leucine-U-C^{14} containing 2,000 cpm per ml, and having an absorbance of 5 at 260 mμ in a 1 cm light path. The latter material was prepared by Dr. Cantoni and Mr. R. Morrison employing a method to be

published. All solutions used in the incubation mixtures were neutralized to approximately pH 7.4.

The pH 5 fraction of rat liver was prepared according to Hoagland *et al.*[15] in the manner prescribed for minimizing base-line exchange. The separation of ATP from PP was effected in the conventional manner.[16] Orthophosphate was determined by the method of Gomori.[17] The measure of exchange used is the specific activity of the ATP expressed as per cent of the value which would have obtained at equilibrium.[15]

Liver extracts for incorporation experiments were prepared essentially according to Keller and Zamecnik.[18] Homogenization was carried out in medium A[18] or in cold isotonic sucrose (0.25 *M*). The supernatant fluid after centrifugation at 15,000*g* is designated the crude extract and abbreviated "C." The upper portion of supernatant fluid after a further centrifugation for 1 hour at 105,000*g* in the Spinco preparative ultracentrifuge is designated "S." The remaining microsomal pellet, after rinsing with medium and suspension by gentle homogenization in the previously employed medium (one-third the volume of the crude extract), is designated "M."

Protein was prepared for counting essentially according to Siekevitz.[19] Where radioactivity in S-RNA was determined, 0.64 *N* perchloric acid was substituted for trichloroacetic acid in the initial washes and the RNA hydrolyzed by heating in 0.96 *N* perchloric acid for 15 minutes at 90°. Protein was removed by low speed centrifugation and a portion of the supernatant fluid neutralized with 2 *N* KOH at 0°. The precipitated $KClO_4$ was removed by centrifugation in the cold and one ml of supernatant fluid dried on a 3 cm diam stainless steel planchet. A separate portion was diluted for measurement of absorbance at 260 mμ in a Beckman spectrophotometer from which the mg of RNA were calculated.[20] Samples were plated at approximately "infinite thinness" as determined from self-absorption curves. Radioactivity was measured with a Nuclear micromil window gas flow counter.

Results.—Demonstration that puromycin inhibts leucine incorporation into protein: When a rat liver extract containing microsomes in addition to the soluble enzymes necessary for amino acid activation and transfer is incubated with ATP, a nucleoside triphosphate-generating system, and radioactive leucine, the protein subsequently isolated is found to be labeled. Previous workers have furnished evidence that this incorporation corresponds to protein synthesis.[13] When low concentrations of puromycin comparable to those required for growth inhibitory activity[5] are present in such a system, the incorporation of amino acid is lowered (Table 1). In this experiment the antibiotic was preincubated with rat liver extract for 5 min at about 23° before addition of labeled leucine and incubation at 37° (see legend). However, when preincubation was omitted the extent of puromycin inhibition of leucine incorporation was identical. Although this evidence fails to suggest a rate-limiting prior conversion of puromycin to an inhibitory compound, the possibility that a metabolite of puromycin represents the true inhibitor is not excluded. Indeed, cleavage of puromycin at the peptide bond yields an aminonucleoside known to be more active against mammary adenosarcomas in mice than puromycin itself.[21] Effects of the aminonucleoside on the rat liver system are currently under study.

To insure that the inhibitory effects observed require the nucleoside structure of puromycin, the antibiotic was submitted to hydrolysis in 1 *N* HCl at 100° for 20

min, twice the time period required for complete cleavage of the glycosidic bond as measured spectrophotometrically by the shift in extinction maximum in 0.1 *N* HCl.[7] The hydrolysate was neutralized, and tested on the rat liver system as shown in Table 1. The absence of inhibition here is consistent with the known absence of biological activity of hydrolysis products of puromycin.[7]

TABLE 1

EFFECT OF PUROMYCIN ON LEUCINE-C^{14} INCORPORATION INTO PROTEIN OF A CRUDE EXTRACT OF RAT LIVER

No.	Puromycin mμM	CPM above background per mg protein	Per cent inhibition
1*	0	0.0	..
2	0	27.2	..
3	5	14.2	48
4	10	9.0	67
5	20	4.4	84
6	200	0.3	99
7	0	27.3	..
8	200†	30.2	0

After 5 min of preincubation at 23° in the absence of added leucine, incubation with labeled leucine was carried out for 30 min at 37° in a final volume of 1.2 ml. The incubation mixture contained: 0.7 ml crude extract in medium A[18], 1.5 μmoles each of ATP and $MgCl_2$, 20 μmoles of phosphoglycerate, puromycin as indicated, and 0.2 μmoles of DL-leucine-1-C^{14} (0.13 μC L-leucine-1-C^{14} corresponding to approximately 7×10^8 cpm). Tube 7 received 10 μmoles of NaCl.

* Tube 1 received 1 ml of cold 0.73 *M* trichloroacetic acid immediately after addition of tagged leucine.

† Puromycin was hydrolyzed for 20 min in 1 *N* HCl at 100° and neutralized with NaOH so that the added solution contributed 10 μmoles of NaCl.

Absence of puromycin effect on ATP-generating systems: In separate experiments, similar to the experiment of Table 1, we have shown that omission of either the energy-generating system or ATP almost entirely eliminates leucine incorporation into protein.

In the experiment of Table 1, added phosphoglycerate and enzymes present in the crude extract were relied upon to regenerate the required nucleotides, i.e., the added ATP as well as endogenous GTP. In other experiments phosphocreatine was provided as the energy source in the presence of added ATP-creatine transphosphorylase. Puromycin was found to inhibit leucine incorporation into protein equally well independently of the energy-generating system used. Since the inhibitory effects of puromycin on leucine incorporation might be attributed to an inhibition of these energy-generating systems, an experimental test of this hypothesis was undertaken. Under the conditions of the leucine incorporation experiments with crude extracts, added ATP disappeared almost completely after 15 min of incubation even in the presence of an energy-generating system. To determine whether the temporary maintenance of the original concentration of ATP was affected by puromycin, it was necessary to perform the ATP determinations after a shorter period of incubation. An incubation time of 3 min proved to be satisfactory. ATP was determined by the direct and specific method of Strehler and McElroy[22] which employs the luminescent system of fireflies.

As can be seen from Table 2, at least 90 per cent of the ATP added is destroyed within 3 min in the absence of a system to replenish it. Either of the two generating systems permit the original level of ATP to be maintained. The slight increase in ATP concentration with phosphocreatine as energy source may represent phosphorylation of ADP present in the crude extract. Levels of puromycin which

TABLE 2

ABSENCE OF PUROMYCIN EFFECT ON ATP-GENERATION

No.	ATP-generating system	Puromycin mμM	ATP found μM
1*	..	0	1.0
2	..	0	0.1
3	Cr-P	0	1.3
4	Cr-P	200	1.2
5*	..	0	1.0
6	..	0	0.0
7	PGA	0	0.8
8	PGA	200	0.9

Incubation was carried out for 3 min at 38° in a final volume of 1 ml. Incubation was started by the addition of crude extract to prewarmed tubes containing the remaining ingredients of the incubation medium. The incubation mixtures of tubes 1, 2, 3, and 4 contained: 25 μmoles of potassium phosphate adjusted to pH 7.5, 50 μmoles of sucrose, 6 μmoles of $MgCl_2$, 0.2 μmole of L-leucine, 1.0 μmole of ATP, and 0.3 ml of crude rat liver extract prepared in 0.25 *M* sucrose. Where the presence of Cr-P for ATP-generation is indicated the incubation mixtures contained in addition 5 μmoles of phosphocreatine and 0.05 ml of ATP-creatine transphosphorylase. The incubation mixtures of tubes 5, 6, 7, and 8 contained 0.2 μmole of L-leucine, 1.0 μmole of ATP, and 0.6 ml of crude rat liver extract prepared in medium A[18]. Where the presence of PGA for ATP-generation is indicated 17 μmoles of phosphoglycerate were included in the incubation mixture. Puromycin was present as indicated. Incubation was terminated by the addition of 1 ml 1.3 *N* $HClO_4$ at 0°. The precipitated protein was removed by centrifugation at 0°. A portion of the superntant fluid was neutralized with KOH to a pH of approximately 7 after addition of sufficient phosphate buffer to prevent overtitration. The precipitated $KClO_4$ was removed by centrifugation at 0° and the supernatant fluid assayed for ATP by the method of Strehler and McElroy.[22]

* Tubes 1 and 5 each received 1 ml 1.3 *N* $HClO_4$ before addition of rat liver extract.

The values of the ATP determinations are expressed as the total number of μmoles found in each incubation mixture.

completely inhibit leucine incorporation into protein do not affect the ability of either generating system to maintain the original level of ATP. These experiments were performed under the conditions of the leucine incorporation experiments. The results therefore appear to exclude the possiblity that the inhibitory effect of puromycin on leucine incorporation into protein is due to an inhibition of systems responsible for ATP-generation.

Absence of puromycin effect on leucine activation: The activation step is the first reaction which leucine undergoes on its pathway into protein. The reaction is conveniently studied by measuring the leucine-dependent incorporation of PP^{32} into ATP in a "pH 5 fraction" of rat liver extract.[15] It may be seen from Table 3

TABLE 3

ABSENCE OF PUROMYCIN EFFECT ON THE RATE OF LEUCINE ACTIVATION BY THE "pH 5 FRACTION" OF RAT LIVER

No.	L-leucine added μM	Per cent PP-ATP exchange† without puromycin	Per cent PP-ATP exchange† with 200 mμM puromycin
1*	5	...	0.4
2	0	2.1	2.2
3	0.1	6.7	6.9
4	5	9.0	9.9

After 5 min of preincubation at 23° in the absence of PP^{32}, but with 5 μmoles of unlabeled PP, incubation with PP^{32} was carried out for 15 min at 37° in a final volueme of 1 ml. The incubation mixture contained: L-leucine and puromycin as indicated, 5 μmoles of PP^{32} (3.1×10^8 cpm), 10 μmoles of KF, 2.4 μmoles each of ATP and $MgCl_2$, 100 μmoles of tris-(hydroxymethyl)-aminomethane adjusted to pH 7.6, and 0.15 ml of "pH 5 fraction."

* Tube 1 received 1 ml of cold 0.73 *M* trichloroacetic acid immediately after addition of PP^{32}.

† Per cent PP-ATP exchange is the specific activity of the ATP expressed as per cent of the value which would have obtained at equilibrium.

that levels of puromycin completely inhibitory for protein synthesis do not affect the leucine-dependent exchange. This is true at a saturating leucine concentration, and, more important, it is also true at a subsaturating leucine concentration

identical to the level of leucine present in the protein synthesis experiment of Table 1. If puromycin were to interfere with the activation of some amino acid other than leucine we would not expect this to affect leucine incorporation into protein as completely as is the case. Therefore, barring a microsome-dependent conversion of puromycin to an inhibitor of leucine activation, the puromycin inhibition of leucine incorporation into protein cannot be attributed to an affect on amino acid activation.

Absence of puromycin effect on the transfer of leucine to S-RNA: The second reaction which leucine undergoes on its pathway into protein is linkage to a specific transfer RNA present in S-RNA. The reaction was studied by analyzing the RNA of the 105,000*g* supernatant fluid for radioactivity subsequent to incubation with labeled leucine. In the absence of microsomes protein does not become labeled (Table 4). Radioactive leucine attached to the S-RNA was liberated from unlabeled protein by treatment with hot $HClO_4$ (see *Methods*).

TABLE 4

ABSENCE OF PUROMYCIN EFFECT ON THE INCORPORATION OF LEUCINE-C^{14} INTO S-RNA

		Radioactivity incorporated into			
		Protein		RNA	
No.	Puromycin mμM	CPM above background per mg protein	Per cent inhibition	CPM above background per mg RNA[20]	Per cent inhibition
C 1*	0	0	..	...	..
2	0	254	..	...	..
3	5	163	36	...	..
4	200	13	95	...	..
S 1*	0	<2	..	140	..
2	0	<2	..	1,350	..
3	5	<2	..	1,520	0
4	200	<2	..	1,460	0

Incubation was carried out for 15 min at 37° in a final volume of 1 ml. The incubation mixture contained: 25 μmoles of potassium phosphate adjusted to pH 7.5, 50 μmoles of sucrose, 0.5 μmoles each of ATP and GTP, 5 μmoles each of phosphocreatine and $MgCl_2$, 0.2 μmoles of L-leucine-U-C^{14} (1.3 μC L-leucine-U-C^{14} corresponding to approximately 7×10^6 cpm), 0.05 ml ATP-creatine transphosphorylase, and puromycin as indicated. Incubation was started upon addition of 0.3 ml of the last component: crude extract C, or supernatant fluid S. These extracts were prepared in isotonic (0.25 M) sucrose.

* Tubes C-1 and S-1 each received 1 ml 0.73 *M* cold trichloroacetic acid immediately after addition of liver extract.

Puromycin was found to have no effect on the extent of labeling of S-RNA. This is true when the antibiotic was present at concentrations that result in essentially complete inhibition of leucine incorporation into the protein of the same rat liver when a crude extract is incubated with radioactive leucine (Table 4).

Under the conditions of our experiments, the endogenous S-RNA becomes labeled very rapidly, the process proceeding to completion within 45 seconds from the start of incubation whether or not puromycin (200 mμmoles per ml) is present. Whether puromycin affects the rate of S-RNA labeling by radioactive leucine remains to be investigated under conditions where this rate is measurable.

Demonstration that puromycin inhibits transfer of leucine from transfer RNA-leucine to microsomal protein: Since puromycin inhibits the over-all incorporation of leucine into protein but apparently neither its activation nor attachment to transfer RNA, we were led to expect an effect of puromycin between transfer RNA-leucine and protein. This expectation was confirmed by the experiment of Table 5. A level of puromycin which reduces the incorporation of free leucine into the protein of a crude extract by 65 per cent causes equivalent inhibition when the starting

TABLE 5

EFFECT OF PUROMYCIN ON THE INCORPORATION OF LEUCINE-C^{14} INTO PROTEIN, COMPARING FREE LEUCINE WITH LEUCINE BOUND TO S-RNA

No.	Initial form of leucine-C^{14}: Free	Initial form of leucine-C^{14}: bound to S-RNA	Puromycin mμM	CPM above background per mg protein	Per cent inhibition
C 1*	+	..	0	0.0	..
2	+	..	0	37.7	..
3	+	..	20	13.3	65
4*	..	+	0	0.0	..
5	..	+	0	19.4	..
6	..	+	20	7.0	64
M 1*	+	..	0	0.0	..
2	+	..	0	39.2	..
3*	..	+	0	0.3	..
4	..	+	0	17.1	..
5	..	+	5	14.6	15
6	..	+	20	9.7	44
7	..	+	200	2.5	87
8	..	+	0	14.9	..
9	..	+	20†	16.6	0

After 5 min of preincubation at 23° in the absence of leucine-C^{14}, incubation with the radioactive component was carried out for 30 min at 37° in a final volume of 1 ml. Each incubation mixture contained: 25 μmoles of potassium phosphate adjusted to pH 7.5, 50 μmoles of sucrose, 0.5 μmoles each of ATP and GDP, 5 μmoles each of phosphocreatine and $MgCl_2$, and 0.05 ml of ATP-creatine transphosphorylase. Tubes labeled C contained 0.25 ml crude homogenate, those labeled M contained 0.2 ml microsomal preparation. These extracts were prepared in isotonic (0.25 *M*) sucrose. Tubes M-3 through M-9 each contained 5 μmoles of unlabeled L-leucine. Where incubation with free leucine-C^{14} is indicated 0.154 μmoles of DL-leucine-1-C^{14} (0.1 μC L-leucine-1-C^{14} corresponding to approximately 5×10^8cpm) was present. Where incubation with S-RNA-leucine-U-C^{14} is indicated the material added contributed about 2,000 cpm, and contained no more than 0.04 mμmoles of S-RNA-leucine as estimated from its absorbance at 260 mμ, assuming a molecular weight of 30,000[23] and that one-twentieth of S-RNA is S-RNA-leucine. Puromycin was present as indicated. Tube M-8 received 1 μmole of NaCl.

* Tubes C-1, C-4, M-1, and M-3 each received 1 ml cold 0.73 *M* trichloroacetic acid immediately after addition of radioactive material.

† Puromycin was hydrolyzed for 20 min in 1 *N* HCl at 100° and neutralized with NaOH so that the added solution contributed 1 μmole of NaCl.

material is leucine bound to its transfer RNA. This result is not due to any release of leucine from S-RNA into the free state since identical incorporation from bound leucine occurs when unlabeled free leucine is present in 25,000-fold excess. When the crude extract is replaced by a microsomal preparation comparable results are obtained. As with the over-all incorporation of free leucine into protein, it can be shown that the partial reaction studied here is insensitive to acid-hydrolyzed puromycin.

These experiments demonstrate that the inhibitory effect of puromycin on leucine incorporation into protein may be accounted for as an inhibition of the transfer of S-RNA-bound leucine into protein.

Discussion.—The wide variety of plant and animal material showing growth inhibition by puromycin suggests that the antibiotic interferes with some major biochemical process not differing in detail from one organism to another. Two considerations call attention to the protein synthetic mechanism as a possible site of action for the antibiotic. First, the observation of Creaser that induced β-galactosidase synthesis in *Staphylococcus aureus* is completely inhibited by low levels of puromycin. Second, the structural resemblance between puromycin and the functional end of apparently ubiquitous intermediates in protein synthesis, the transfer RNAs.

Experiments with labeled leucine conform to our expectation that puromycin interferes with protein synthesis. The site of this interference appears to be largely, if not entirely, beyond the formation of transfer RNA-leucine. Our experiments

on the two reactions necessary for leucine attachment to transfer RNA do not permit us to exclude a puromycin effect on either of these reactions which is dependent upon the presence of microsomal particles. However, the fact that we can quantitatively account for inhibition of the over-all reaction by the inhibitory effect of puromycin on a subsequent step shows that at least the early reactions are not inhibited by puromycin to the point where they become rate-limiting.

It is interesting to note that administration of the aminonucleoside of puromycin to rats induces a nephrotic syndrome.[24] Examination of the effects of the aminonucleoside on amino acid incorporation may suggest a biochemical basis for this observation. Such studies may also shed light on whether puromycin must be degraded, e.g., to the aminonucleoside, or built into some cellular metabolite, before it becomes inhibitory. If they occur, these reactions must be relatively fast since they appear to be complete before appreciable leucine has been incorporated into protein, a process which itself goes to completion within a few minutes.

Recent evidence indicates that reaction 3 of the protein synthetic pathway may be resolved into a GTP-stimulated passage of transfer RNA-amino acid (or some portion thereof bearing the amino acid) into the microsome,[13, 25–27] and a second step which brings about the incorporation of the bound amino acid into protein. It is hoped that the site of puromycin action may be further localized by separate examination of these two steps.

Two additional antibiotics appear to resemble puromycin in their site of action. Experiments by Lacks and Gros[28] and by Hopkins[29] suggest that chloramphenicol inhibits amino acid transfer from S-RNA to protein. A recently reported inhibition by dihydrostreptomycin of amino acid incorporation into mycobacterial protein *in vivo*[30] and *in vitro*,[31] again appears to be due to a failure in the protein synthetic pathway at this step.[31] Examination of other antibiotics, in particular those for which a cross-resistance develops to the antibiotics mentioned above, may reveal additional inhibitors acting at a similar site.

Summary.—(1) Structural similarities between the broad-spectrum antibiotic puromycin (6-dimethylamino-9-[3′-deoxy-3′-(*p*-methoxyl-*L*-phenylalanylamino)-β-D-ribofuranosyl]purine) and the amino acid-bearing end of S-RNA are discussed. (2) Puromycin was found to inhibit the incorporation of leucine-C^{14} into protein in a cell-free preparation from rat liver. (3) Puromycin does not inhibit the rate of ATP-generation by either of two enzyme systems used in the leucine incorporation experiments. It does not inhibit the rate of leucine activation by the "pH 5 fraction" of rat liver. No puromycin inhibition of the extent of S-RNA labeling with leucine-C^{14} could be detected. (4) The transfer of leucine-C^{14} from S-RNA-leucine-C^{14} to protein is inhibited by puromycin. The extent of this inhibition is considered sufficient to account for the inhibition by puromycin of leucine incorporation into protein. (5) Observations of other workers which suggest a similar site of action for the antibiotics chloramphenicol and dihydrostreptomycin are briefly discussed.

Of the many people to whom we are indebted, we wish to thank particularly Dr. Giulio L. Cantoni for his generous gift of S-RNA-leucine-C^{14} and for helpful discussions. We also wish to express our gratitude to Dr. Herman M. Kalckar for his advice and encouragement.

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The following abbreviations are used: (S-)RNA, (soluble) ribonucleic acid; AA, amino acid; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine-5′-phosphate; AMP-AA, amino acid adenylate; E, amino acid activation and transfer enzyme; PP, pyrophosphate; Ms, microsome or ribosome; GTP, guanosine triphosphate; GDP, guanosine diphosphate.

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‡ Contribution No. 272 of the McCollum-Pratt Institute.

[1] Puromycin was formerly known as Achromycin, the trade-name of Lederle Laboratories Division, American Cyanamid Company. Stylomycin is the current trade-name of Lederle Laboratories for puromycin. Achromycin is the trade-name of Lederle's brand of tetracycline.

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INHIBITION OF THE ENZYMIC OXIDATION OF DPNH BY STEROID HORMONES

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The role of the steroid hormones in the regulation of cell metabolism, although of considerable biological importance, is not well understood. Progress has been made by two groups of investigators[1, 2] who have ascribed to some steroids the function of catalyzing the enzymic transfer of hydrogen between di- and triphosphopyridine nucleotides, although the mechanism of this reaction is controversial.[3, 4]

The present communication describes an apparently unrelated general property possessed by a number of hormonally active steroids, that is, their ability to inhibit, at low concentrations, the oxidation of DPNH catalyzed by enzymes derived from both animal and microbial sources.

Materials and Methods.—DPNH, TPNH, steroids, diethylstilbesterol, cytochrome C, α-tocopherol, and *n*-butyl stearate were commercial preparations. Samples of ubiquinone (Q 275, obtained originally from Merck and Company) were gifts from Drs. T. C. Stadtman and L. Corwin. Erlich and Sarcoma 37 ascites tumor-bearing mice were kindly supplied by Dr. Morris Belkin of the National Cancer Institute. Tumor cells were harvested 4 and 6 days, respectively, after inoculation.

Saccharomyces fragilis (ATCC #10022) was grown aerobically in a 5 per cent glucose salts solution[5] for 36 hours at room temperature. *E. coli*, strain B, and *B. subtilis* (ATCC #465) were grown overnight, with shaking, at 37° in a medium consisting of Difco nutrient broth 8 gm., sodium chloride 4 gm., dissolved in 1 liter of tap water.

Preparation of DPNH Oxidases.—Fractions from the tissues of male Sprague-Dawley rats were prepared by the procedure used by Lehman and Nason to obtain the particulate DPNH-cytochrome C reductase system.[6] Organs were homogenized in a volume of 0.1 *M* phosphate buffer, pH 7.5, equal to 10 times the wet weight of tissue. The supernatant layer, obtained after centrifugation at 3000 $\times$ *g* for 15 minutes, was dialyzed against 10 volumes of 0.01 *M* phosphate buffer, pH 7.5 for one hour. The dialyzed preparation was then centrifuged at 100,000 $\times$ *g* for 30 minutes and the resulting pellet was resuspended in one-tenth its original volume of 0.1 *M* phosphate buffer, pH 7.5. All of the foregoing operations were carried out between 0 and 5°. Microsomal and mitochondrial preparations were also made from various tissues by the technique of Hogeboom[7] using 0.25 *M* sucrose-0.001 *M* EDTA as the medium for all operations.

Tumor and yeast cells, harvested by centrifugation, were resuspended in the sucrose-EDTA solution. The suspensions were mixed with an equal volume of glass beads (Minnesota Mining & Manuf. Co. #150 75μ) and fragmented in the Nossal disintegrator[8] at -5°C. Tumor cells were treated for 30, and yeast cells for 60 seconds, in 15 second periods separated by intervals of 15 seconds. Particles corresponding to mitochondria and microsomes were then isolated by differential centrifugation.[7]

Bacterial cells, harvested by centrifugation, were washed once and resuspended in distilled water. The suspensions were subjected to high frequency sound waves

for 15 minutes in a Raytheon 9 Kc sonic oscillator maintained near 0°. The disrupted cells were then centrifuged at 13,000 × *g* for 10 minutes to remove cell debris. The supernatant layer was re-centrifuged at 100,000 × *g* for one hour and the pellet was re-suspended in a volume of distilled water equal to half the original volume centrifuged. The clear supernatant fraction was also saved and tested as described below.

Microsomal cytochrome reductase, prepared from rabbit liver according to the method of Velick and Strittmatter[9] was carried through to the lyophilized alcohol extract step. Cytochrome C was reduced by hydrogen over Pd and asbestos.[10]

Emulsions of tocopherol, ubiquinone, *n*-butyl stearate, and butter were prepared according to Nason and Lehman.[11]

Steroids and diethylstilbesterol were added to reaction mixtures as solutions in 50 per cent (by volume) propylene glycol. In experiments where the effects of tocopherol, other lipids, or steroids were tested, controls were run which contained the appropriate medium.

Enzyme Assays.—Oxidation of DPNH was estimated by measuring the decrease in optical density at 340 mμ in the Beckman model DU spectrophotometer at room temperature in 3 ml silica cells of 1 cm light path. The initial rate, determined during the first 5-minute period, was expressed as the change in optical density/minute. The rates were constant until the DPNH was virtually exhausted. The molar extinction coefficient of reduced DPN^+ was considered to be 6.22×10^3 l/mole cm.[12]

DPN cytochrome C reductase activity was measured by the method described by Lehman and Nason[6] in which substrate quantities of cytochrome C were added to the reaction mixture together with sufficient KCN (10^{-3} *M*) to prevent its oxidation. The course of the reductase reaction was followed by observing either DPNH oxidation at 340 mμ, or cytochrome C reduction at 550 mμ. Succinate cytochrome C reductase was assayed in the same way[6] by measuring the rate of reduced cytochrome C formation when succinate was added to the reaction mixture.

Microsomal cytochrome reductase activity was determined from the rate of DPNH oxidation with $Fe(CN)_6^{-3}$ as the electron acceptor.[9] This value was corrected for the nonenzymic oxidation of DPNH by ferricyanide.

Cytochrome oxidase was measured spectrophotometrically by following the oxidation of reduced cytochrome C at 550 mμ.[10]

Results.—Figure 1 shows the effect of progesterone on the enzymic oxidation of both DPNH and TPNH, catalyzed by microsomes prepared from rat kidney. Curve A depicts the oxidation of DPNH in the absence of added steroid, and Curve B illustrates that, in the presence of 2×10^{-5} *M* progesterone, this rate was drastically reduced. Curves C and D demonstrate that the same concentration of steroid did not affect TPNH oxidation. This inhibitory action of progesterone appeared to be catalytic since 0.05 micromoles of hormone produced an inhibition corresponding to 0.35 micromoles of DPNH. This suggested that the reduction of DPN by the steroid could not account for the difference between the experimental and control cuvettes. In support of this, when DPN was incubated with progesterone and enzyme under the same conditions, no reduced DPN was formed.

The effects of two steroids, progesterone and deoxycorticosterone, on the DPNH oxidase reaction from a variety of sources were examined and the results are given

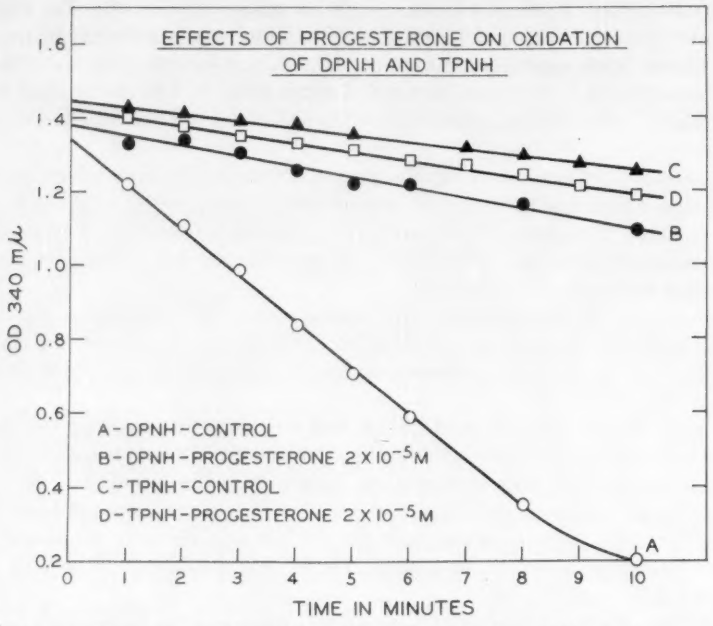

FIG. 1.—Cuvettes A and B contained 0.1 ml of a suspension of kidney microsomes equivalent to 25 mg of original wet weight of tissue, tris buffer, pH 7.25, 0.01 M, $MgCl_2$ 2×10^{-3} M, KCl 7.5×10^{-6} M, sucrose 0.12 M, DPNH 4×10^{-4} M, and 50 per cent propylene glycol 0.1 ml with or without added steroid in a volume of 2.5 ml.

Cuvettes B and C were identical except 0.2 ml of the microsomal suspension was used and TPNH was substituted for DPNH.

in Table 1. Higher steroid concentrations were used with the microbial enzymes than with the animal preparations. The reaction catalyzed by particles obtained from liver, kidney, heart, skeletal muscle, and the tumors, was strongly inhibited by both compounds at 2×10^{-5} *M*. With mammalian tissue, virtually identical results were obtained with either mitochondrial[13] or microsomal preparations, and there was no significant difference whether phosphate buffer or sucrose-EDTA was used.

The rate of DPNH oxidation, catalyzed by the particles of *S. fragilis* which sedimented at 9,000 $\times$ *g* (*Mitochondria* in Table 1), was reduced almost 70 per cent by 5×10^{-4} *M* progesterone. The sediment obtained at 100,000 $\times$ *g* was not inhibited as strongly, however.

The DPNH oxidase reactions of *E. coli* and *B. subtilis* were also studied. The soluble enzymes, i.e., those not sedimenting at 100,000 $\times$ *g* in 1 hour, were inhibited 35–50 per cent by either progesterone or DOC at 5×10^{-5} *M*.

Having thus demonstrated that progesterone and DOC have a general inhibitory effect on DPNH oxidation, it was important to ascertain both the number of steroids which possess this inhibitory capacity and the extent to which the various steroids are inhibitory. Data releveant to these points are presented in Table 2, in which the K_i values (obtained by the method of Dixon and Webb[14]) are shown

for nine steroids with enzymes derived from three tissues of the rat. These represent concentrations of inhibitors which produce half-maximal inhibition and are assumed to be proportional to the affinities of the vulnerable enzymes for the steroid

TABLE 1

EFFECTS OF PROGESTERONE AND DEOXYCORTICOSTERONE ON DPNH OXIDASES

Enzyme Source in ml	ΔO.D.$_{340}$/min. × 10^2			Inhibition	
	Control	Progesterone	DOC	Progesterone	DOC
		2×10^{-5} *M*		Per Cent	
Liver[1] 0.1	1.25	0.35	0.78	72	41
Kidney[1] 0.05	6.37	1.68	3.85	74	39
Heart[1] 0.05	3.85	1.60	2.76	58	28
Skeletal muscle[4] 0.02	3.78	1.62	2.40	57	36
Sarcoma 37[1] 0.05	1.53	0.36	..	77	..
Ehrlich ascites[1] 0.05	1.73	0.38	..	78	..
S. fragilis[2] 0.1	7.70	2.38*	4.20*	69	45
E. coli[3] 0.1	8.3	4.08†	4.32†	51	48
B. subtilis[3] 0.1	3.25	2.13‡	1.98‡	35	39

Experiments 1–3 and 5–9 were conducted as described under Figure 1, cuvettes A and B, except for the volume of the enzyme used and the substitution of 2×10^{-4} *M* DPNH. In experiment 4, the reaction mixture contained phosphate buffer, pH 7.5, 0.09 *M*, cytochrome C 0.04 per cent, DPNH 2×10^{-4} *M*, 50 per cent propylene glycol 0.1 ml alone or with steroid. All the reaction volumes were 2.5 ml.

* Steroid concentration 5×10^{-4} *M*.

† Steroid concentration 5×10^{-5} *M*.

[1] Microsomes, 1 ml equal to 250 mg original wet weight of tissue.

[2] Mitochondria, 1 ml equal to 250 mg original wet weight of tissue.

[3] 100,000 × *g* supernate, 1 ml equal to 125 mg original wet weight of tissue.

[4] Fraction III of Lehman and Nason,[6] 1 ml equal to 1 gm. of original wet weight of tissue.

inhibitors. In every case progesterone and the synthetic steroid analogue, diethylstilbesterol, were the best inhibitors with K_i's in the range of 8×10^{-7} to 10^{-5} *M* depending on the organ. Deoxycorticosterone, testosterone, estradiol, and dihydrotestosterone were next with values in the neighborhood 5×10^{-5} *M*. The corticoids, cortisone, corticosterone, and the physiologically inactive dihydrocortisone, were least potent, with constants from 2×10^{-5} to 8×10^{-4} *M*. In general, the kidney was the most responsive of the organs tested.

TABLE 2

EFFECTIVENESS OF STEROIDS AS INHIBITORS OF DPNH OXIDATION

Steroid	K_i, M.		
	Muscle	Liver	Kidney
Corticosterone	6.5×10^{-5}	8×10^{-5}	2.6×10^{-5}
Deoxycorticosterone	3×10^{-5}	1.3×10^{-5}	1×10^{-5}
Progesterone	1.6×10^{-5}	6.4×10^{-6}	3.2×10^{-6}
Estradiol	3.7×10^{-5}	*1.4×10^{-5}	..
Diethylstilbesterol	6.4×10^{-6}	6.5×10^{-6}	8×10^{-7}
Testosterone	3.2×10^{-5}	*4×10^{-5}	..
Dihydrotestosterone	2.8×10^{-5}	*8×10^{-5}	..
Cortisone	5×10^{-4}	*1.2×10^{-4}	..
Dihydrocortisone	8×10^{-4}	*3.8×10^{-4}	..

Reactions were carried out as described in Table 1, experiment 4, except that the concentrations of the steroids were varied. The experiments marked * were conducted as described in Table 1, experiments 1–3.

In addition to those listed in the table, the following compounds were tested with liver microsomes and found to produce substantial inhibition: cortisol, Δ^1 cortisone, 1,4-androstadiene-3,17-dione, 4-androstene-3,17-dione, androstane-3,17-dione, 5β-androstane-3,17-dione, 19-nor testosterone, 11β-hydroxyprogesterone, 11α-hydroxyprogesterone, 17α-hydroxyprogesterone, 5α-pregnan-3β-ol-20-one, and pregenolone. Several steroids, however, had no effect on DPNH oxidation. These were tetrahydrocortisone, cholesterol, ergosterol, digitoxin, and digoxin.

The DPNH oxidase reaction is composed of many individual steps: the electrons from the reduced pyridine nucleotide are passed through a flavoprotein, the cytochromes and eventually, to oxygen. It was obviously of interest to localize the steroid inhibition as precisely as possible. To this end, reduced cytochrome C was prepared and its oxidation tested to give a measure of cytochrome oxidase activity in skeletal muscle. No inhibitory effect of steroid hormones was noted.

The span between DPNH and cytochrome C in particles from skeletal muscle was then examined directly. Figure 2 illustrates the results of these experiments.

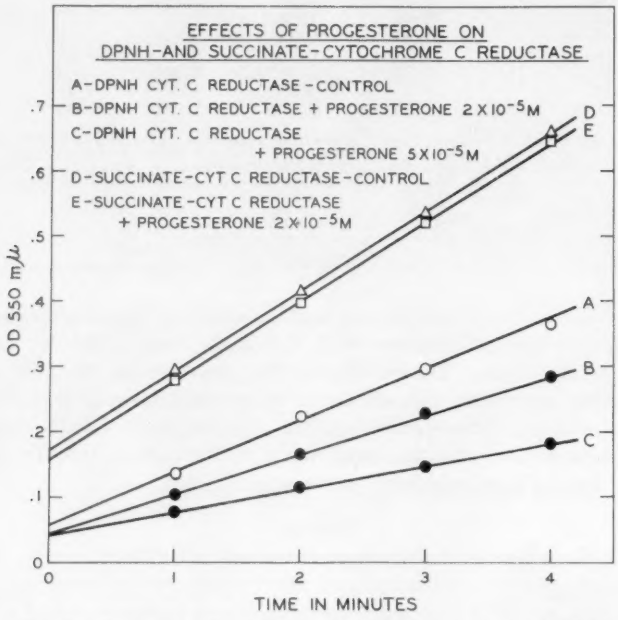

FIG. 2.—Cuvettes A, B, and C were identical to experiment 4, Table 1, with addition of 1×10^{-3} M KCN and the substitution of 0.08 per cent cytochrome C.

Cuvettes D and E contained 0.1 ml of a suspension of heart mitochondria, equivalent to 25 mg of wet weight of tissue, phosphate buffer pH 7.5, 0.09 M, KCN 1×10^{-3} M, cytochrome C 0.09 M, KCN 1×10^{-3} M, cytochrome C 0.08 per cent, sodium succinate 8×10^{-3} M, and 50 per cent propylene glycol 0.1 ml alone or with added steroid.

Here the reduction of cytochrome C, in the presence of 10^{-3} *M* cyanide, was followed at 550 millimicrons. Two and 5×10^{-5} *M* progesterone exerted a considerable inhibitory effect on the enzymic reduction of cytochrome C when DPNH was the hydrogen donor. It should be noted, however, that the degree of progesterone inhibition observed here was considerably less than when its effect on DPNH oxidation was measured in the absence of cyanide. This could be explained by the concurrent observation that the rate of DPNH oxidation was itself inhibited as much as 90 per cent when cyanide was added, even in the presence of substrate amounts of cytochrome C. This was due, perhaps to the accumulation of reduced cytochrome

C behind the cyanide block. It appeared, therefore, that a different reaction became rate-limiting in the DPNH-cytochrome C reductase sequence in the presence of cyanide. The reductase would consequently appear less sensitive to inhibition by steroid under these conditions. This interaction of steroids with the DPNH-cytochrome C reductase system could account for the observed inhibition of the DPNH oxidase reaction, at least in skeletal muscle. Since both the DPNH- and succinate-cytochrome C reductases may transfer electrons to cytochrome C via cytochrome b, it was of interest to determine whether the reduction of cytochrome C by succinate would likewise be affected by steroids. The results of an experiment

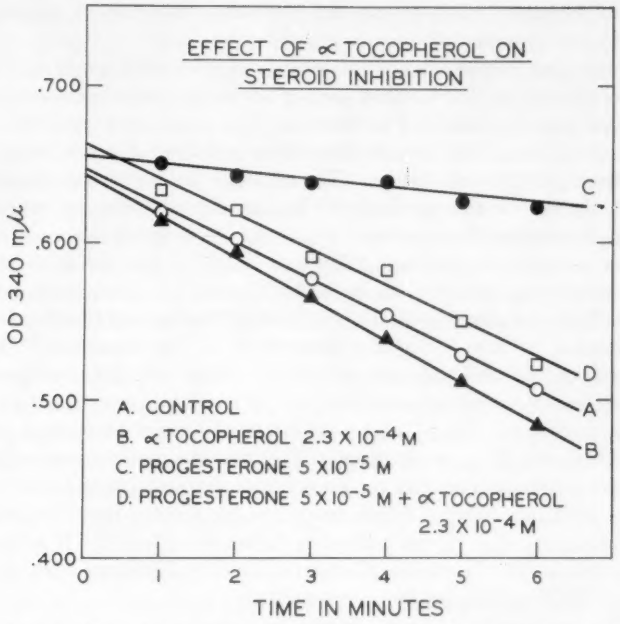

FIG. 3.—The reaction conditions were as described for experiment 4, Table 1, with additions as noted.

to test this are also illustrated in Figure 2. No inhibitory effect of progesterone on cytochrome reduction was noted when succinate was the hydrogen donor.

Recently, several investigators have been concerned with the role of lipids in the DPNH-cytochrome C reductase system.[11, 15–21] For this reason we have investigated the effect of α-tocopherol on steroid inhibition of the DPNH-cytochrome C reductase of skeletal muscle. In Curve A of Figure 3, the rate of DPNH oxidation is plotted, and in Curve B a similar experiment is shown run in the presence of 2.3×10^{-4} M α-tocopherol, which did not affect the control rate significantly. In Curve C, the inhibition due to progesterone is noted and in Curve D, this inhibition has been completely reversed by the further addition of 2.3×10^{-4} M α-tocopherol. This reversal by α-tocopherol was also demonstrated with bacterial preparations. In other experiments with the muscle enzyme, kinetic analysis has indi-

cated that inhibition by progesterone was competitively reversed by α-tocopherol.

Since Donaldson and Nason have found that, in addition to α-tocopherol, various other compounds can reactivate iso-octane-extracted preparations of DPNH-cytochrome C reductase,[21] we have tested some of these, i.e., *n*-butyl stearate, menadione, and butter. All of these substances were found to release the steroid inhibition. Ubiquinone, however, did not alter the response to steroid under the conditions of our assay.

In view of the sensitivity of liver microsomes to inhibition by the steroids, and because the major route of electron flow in these particles is thought to be through the flavoprotein, microsomal cytochrome reductase,[9] this enzyme was prepared and assayed directly. Progesterone did not reduce the rate of enzymic DPNH oxidation by ferricyanide.

Discussion.—Our results showed that steroid hormones could act as potent inhibitors of the enzymic oxidation of DPNH while the oxidation of neither TPNH nor succinate was impaired. Furthermore, this action was catalytic since low concentrations of the hormones inhibited the oxidation of much larger amounts of the reduced pyridine nucleotide. The catalytic nature of the steroid effect is clearly desirable in substances which are biologically active in low concentrations.

Recently, it has been demonstrated that some steroids can act as coenzymes for the transfer of hydrogen between DPN and TPN,[1, 2] but the actual manner in which the steroids participate is the subject of dispute.[3, 4] Also, at the present time there is insufficient evidence to decide whether this biochemical function is an important explanation for any of the biological effects of the hormones.[22] Regardless of the status of this problem, our results are almost certainly independent of a steroid-mediated transhydrogenase reaction. If transhydrogenation were involved, it could occur between the substrate, DPNH, and a small amount of endogenous TPN, which would then be oxidized. The steroids would necessarily have to function here as inhibitors rather than activators of transhydrogenation in order to explain the inhibitory effect. Furthermore, we have noted that in every preparation examined (e.g., Fig. 1), the rate of oxidation of added TPNH is considerably lower than that of DPNH, making the participation of TPN and a transhydrogenation in the DPNH oxidase reaction very unlikely.

Although equally unlikely, it might also be argued that our results were due to steroid activation of transhydrogenation between TPNH and DPN^+. The former would have to arise from the reduction of a catalytic amount of TPN by a large amount of endogenous substrate. This minute amount of TPNH could, in the presence of a steroid mediated transhydrogenation, constantly regenerate DPNH, making it appear as though the steroid were inhibiting the oxidation of DPNH. This possiblity is excluded by our findings that in the presence of DPN^+ and steroid no DPNH is formed, even in the presence of cyanide.[23] These considerations, then, make it highly improbable that the participation of steroids (or diethylstibesterol) in the present effect is as a result of inhibition or, for that matter, stimulation of the transhydrogenase reaction.

It seems very unlikely, furthermore, that metabolic alteration of the steroids is necessary for their inhibitory action, because when DPN was incubated with progesterone, no reaction was detected, although under the same conditions, steroid strongly inhibited DPNH oxidation. Also, the variety of inhibitory steroids, and

especially the affectiveness of diethylstilbesterol, makes it inconceivable that a single type of metabolic transformation could produce the observed effect.

Finally, it is significant that both the intracellular and tissue distribution of steroid metabolizing enzymes previously investigated is quite different from that of the DPNH-oxidase reaction.[24, 25]

In the present study, the detailed mechanism of steroid action was examined only in rat skeletal muscle preparations, although it seems reasonable that the same mechanism operates in other tissues as well. The data point to the DPNH-cytochrome C reductase reaction as the site of the inhibition. The latter, however, is a complicated reaction sequence and neither the components of the system nor the exact route of electron transfer from reduced pyridine nucleotide to cytochrome C is fully understood. Because of this it is impossible to say precisely where the steroid is working. It seems relevant that lipids have been implicated in electron transport, although this question is still unsettled.[15-21] The fact that α-tocopherol overcame the steroid inhibition suggests that the same step is involved which was inhibited by iso-octane extraction in the experiments of Nason and his collaborators.[11] This is not necessarily the case since iso-octane extraction inactivated the succinate-, as well as DPNH-cytochrome C reductase, and in both cases the activities could be restored by α-tocopherol.[11] Our results showed that progesterone did not affect the succinate reaction. Furthermore, there was no α-tocopherol stimulation of iso-octane-extracted DPNH oxidase from bacteria,[11] while we have found steroid inhibition of this bacterial oxidase reaction and its reversal by α-tocopherol.

One of the components of the DPNH-cytochrome C reductase reaction is a flavoprotein.[26] It is, therefore, of interest that steroids have been found to inhibit various flavoproteins,[27] but in much higher concentrations than those used in the present study. It may be especially relevant to note that the soluble, highly purified DPNH cytochrome C reductase of heart muscle can be inhibited by prolonged incubation with high concentration (3×10^{-3} M) of deoxycorticosterone,[28] but is not affected by cortisone. There is, therefore, no obvious connection between these results and our findings.

As steroids did not appear to inhibit the DPNH oxidases by virtue of alteration of the steroids themselves, we must conclude that their inhibitory effects are exerted through physical interaction with some undetermined component of the complex enzyme system. An examination of the structures of the effective steroid inhibitors does not reveal any obvious common properties. Compounds which are both saturated and unsaturated in the A ring, as well as the benzenoid female hormones, are active; and polarity, likewise, does not seem to be important. Despite this, however, there is an enormous variation in effectiveness among the various steroids, which is attested to by their K_i values which range from 8×10^{-7} for stilbesterol, to cholesterol which was completely ineffective.

It is obviously important to consider what, if any, physiological significance may be attached to the inhibition of DPNH oxidase, and various properties of this steroid-enzyme interaction suggest that this effect might indeed be biologically significant. First, the high affinity of the DPNH-cytochrome C reductases for the steroids, especially progesterone, estrogen, and stilbesterol, implies that part of the action of these hormones may be as a result of the effect reported here. It is note-

worthy that in mammalian cells the DPNH oxidase reaction is confined to particles which are rich in lipids,[29] and, as Lynn[30] has demonstrated, testis microsomes, because of their high lipid content, concentrate steroids. Therefore, even at low extracellular hormone levels, the concentration at the possible site of action might be considerably higher.

Especially interesting is the action of diethylstilbesterol. This compound, although not a steroid, is structurally very similar to estradiol[31] and has a steroid-like action *in vivo;* and the fact that it, like the steroids, also inhibits these enzyme preparations argues in favor of the biological significance of the effect reported herein.

Since organized biological systems have an amazing capacity to amplify and elaborate on a disturbance at the molecular level, it is not possible to predict what the macroscopic manifestations of such an effect would be. Be that as it may, there are several intriguing possibilities. One of these is the mechanism of progesterone action. The physiological effects of this hormone are seen principally in organs which have been previously stimulated by estrogens. Hollander has studied an enzyme which catalyzes the oxidation of DPNH by molecular oxygen and is stimulated by phenols.[32] It is virtually absent in the uteri of spayed animals, but a single dose of estrogen produces a large increase in its level. If the role of progesterone is actually to inhibit the flow of electrons from DPNH through the cytochrome system, as suggested by our data, the presence of an estrogen-induced alternate pathway for DPNH oxidation might be significant physiologically.

Another possible biological correlation pertains to the almost universal property of steroids to inhibit cell growth. Tumors,[33] tissue culture cells,[34] and microorganisms[35] are affected, and it seems reasonable to suppose that at least some of these inhibitory affects might be the result of the inhibition of DPNH oxidation.

Finally, even though the actual role of α-tocopherol in DPNH-cytochrome C reductase has been disputed,[19] and we cannot discuss with assurance the possible physiological connection between the hormones and a lipid cofactor, it might be pertinent that several of the manifestations of vitamin E deficiency, i.e., fetal resorption in the female and testicular atrophy and sterility in the male, could be related to alterations in steroid hormone action.

From the physiological standpoint, it is somewhat disturbing that all the organs tested seemed to be almost equally responsive to such hormones as stilbesterol or progesterone. It is, therefore, difficult to explain the tissue-specific physiological responses to different steroids. It may be, however, that permeability factors, etc., impose a greater specificity when intact cells are exposed to the hormones. Another important variable might be the degree to which cytochrome C reductase is rate-limiting in cell metabolism.

Summary.—Low concentrations of a large number of steroid hormones and diethylstilbesterol inhibited DPNH oxidation by enzyme preparations from both mammalian and microbial sources. Neither TPNH nor succinate were similarly affected, however.

In skeletal muscle, the site of steroid inhibition proved to be the DPNH-cytochrome C reductase reaction and α-tocopherol and other compounds could competitively reverse the steroid effect.

We should like to acknowledge the devoted expert assistance of Mrs. Janet Munday and Mr. Irwin J. Cowley.

Note added in proof: After this manuscript was submitted for publication, a letter appeared in *Nature* (P. K. Jensen, 184, No. 4684, 451) in which inhibition of DPNH oxidation in heart sarcosomes by corticosterone, cortisol, 11-deoxycorticosterone, 17-hydroxy-11-deoxycorticosterone, and cortisone was reported.

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*MAXIMUM GROWTH RATES OF CHLORELLA IN STEADY-STATE AND IN SYNCHRONIZED CULTURES**†

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Communicated by Sterling B. Hendricks, October 21, 1959

Multicellular organisms and steady-state microbial populations consist of cells in all possible developmental stages. In such populations some cells are vigorously growing, others are preparing to divide, and stll others are actually in the process of cell division. Environmental conditions necessary for the optimum development of one stage in the life history of the cell may be totally inadequate or even inhibitory for growth during another stage. Two paramount processes in the growth and development of cells are the accumulation of cell material and cell division. In autotrophic algal cells the accumulation of cell material depends directly on photosynthesis and consequently on light.

In Chlorella pyrenoidosa cell division does not depend on light in the sense that after accumulation of cell material a cell can divide in complete darkness. In synchronized algal suspension the accumulation of cell material can be separated in time from cell division. The studies reported here were designed to determine the maximum rates of growth attainable during the period of synthesis and accumulation of cell material, and to explore some of the factors which might be stimulatory to this phase of life history but inhibitory to cell division.

Experimental.—The work was done with the green alga, Chlorella pyrenoidosa, high-temperature strain 7-11-05.[1] Details of the experimental technique have been described in previous publications.[1–4] Two series of experiments were conducted. In one series cells were grown in a continuous culture apparatus[5] under constant conditions of temperature, illuminance, and nutrient supply. As the cells grew during the experiment the optical density of the algal suspension was photometrically maintained at a constant level by automatic dilution with fresh medium. Thus, the illuminance experienced by individual cells was kept at a constant level. Incandescent lamps placed in four banks around the growth chamber served as a source of light. The light intensity at the surface of the growth chamber measured with model 756 Weston Illumination Meter provided with a quartz filter was 1,000 ft-c.

In the second series of the experiments algal suspensions were grown in test tubes containing a constant volume of medium. As a source of light day-light, cool-white fluorescent lamps were placed in two banks at the opposite sides of a thermostatically controlled plexiglass water-bath. The light intensity measured at the level of the culture tubes was 2,000 ft-c. As the cells grew the optical density of the algal suspensions increased and the illuminance, as experienced by the individual cells, declined. However, it has been established previously that the growth rate remained exponential for the duration of the experiments. This observation was taken as an indication that the decrease in the illuminance as the result of cell growth did not become limiting to growth under these conditions. It should be emphasized that the light intensities measured in the two series of experiments are not directly comparable because of the difference in the light source, the geometry of illumina-

tion, the optical properties of the glass containers, and the thickness of the layers of algal suspensions.

Synchronization of cell division was achieved by a light:dark regimen.[2,6] After 9 hr of light cells were given 15 hr of darkness. In the dark all cells which were large enough divided, but in the absence of organic carbon they were prevented from growing until after they were exposed to light. After 2–3 dark-light cycles up to 99 per cent of cells were in the stage of small daughter cells at the beginning of the light period. Growth started as soon as lights were turned on. Under the conditions of these investigations the life cycle of the strain 7-11-05 took 9 to 10 hr. During this time no cell division took place in synchronized cultures.

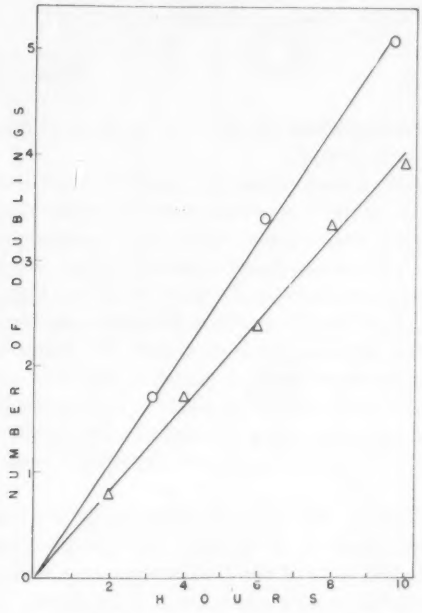

FIG. 1.—Growth of Chlorella pyrenoidosa, strain 7-11-05 as a function of time. Triangles, steady-state suspension; circles, synchronized suspension. Grown in continuous culture apparatus at 1,000 ft.-c.

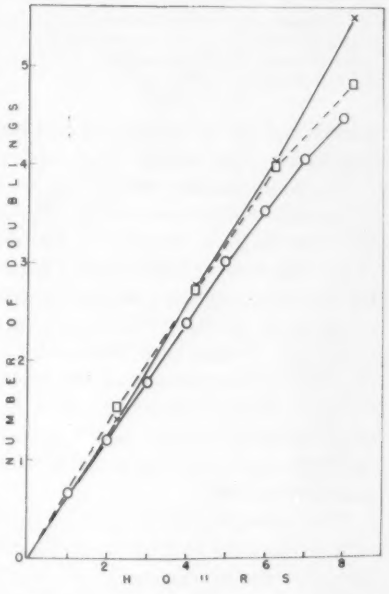

FIG. 2.—Growth of synchronized suspension of Chlorella pyrenoidosa strain 7-11-05 as a function of time. Crosses, dry weight; circles, optical density; squares, packed cell volume. Grown in constant volume of medium, in test tubes.

Results and Discussion.—Representative growth curves for steady-state and synchronized suspensions grown in the continuous culture apparatus during the first series of experiments are compared in Figure 1. The logarithmic shape of the time-course curves indicates that growth was exponential during the first 8 hr of the experiment. This was the period prior to cell division in the synchronized cultures. During 8 hr the cell substance of the steady-state suspension had increased 3.3 times and that of the synchronized suspension had increased 4.7 times.

For synchronized cultures grown in the test tubes during the 2nd series of experiments growth was determined in terms of optical density, packed cell volume, and dry weight (Fig. 2). This was done to insure that there was no error in plotting

growth as a result of qualitative changes in the optical characteristics of the cultures. The increase in optical density for 8 hr was 4.5, in packed cell volume 4.7, and in dry weight 5.3.

The progress that has been made in raising the multiplication rates in algae can be summarized by comparison of the performance of the steady-state and synchronized suspensions of the high-temperature strain with that of the low-temperature, Emerson strain (Table 1). Emerson's strain has been used in a number of investi-

TABLE 1

GROWTH OF ALGAE IN STEADY-STATE AND SYNCHRONIZED SUSPENSIONS

Suspension	Strain	Doubling time, hr.	Number of doublings for 8-hr period	If calculated for 24-hr period Number of doublings	If calculated for 24-hr period Increase, -fold
Steady-state	Low-temperature	8	1	3	8
Steady-state	High-temperature	2.5	3.3	10	1,000
Synchronized	High-temperature	1.5	5.3	16	65,000

gations and can be considered as a standard to which the different levels of multiplication in other strains can be conveniently related.

The minimum generation time, or doubling time, under the optimal conditions for growth of the steady-state suspensions of the low-temperature strain has long been known to be near 8 hr. The doubling time for the steady-state suspensions of the high-temperature strain is 2.5 hr. This means that the number of doublings for the 24-hr period is 3 for the low-temperature strain, and close to 10 for the high-temperature strain. The increase in mass of the algal cells is 8-fold for the low-temperature algae and 1000-fold for the high-temperature algae. By using a synchronized suspension of the high-temperature strain a doubling time can be obtained which is equal to 1.5 hr. As mentioned before this will give 5.3 doublings of cell material in 8 hr. If this rate could be sustained for the entire 24 hour period, the number of doublings would be equal to 16 and the increase in dry weight would amount to 65,000.

The increased growth rates in the absence of cell division gave reason to suspect that strong light might have a detrimental effect on cell division and through it on growth. In the suspensions of synchronized cells of the high-temperature strain cell division usually starts after 9 to 10 hr of growing under optimal conditions of temperature and light intensity. However, when the time for cell division arrives cells do not divide simultaneously. Individual cells divide after different periods of growth. In an algal suspension as a whole, cell divisions are taking place throughout an elapsed time of 8 to 9 hr if the culture remains in the light. If a suspension of cells about to begin cell division is transferred into darkness, then the time necessary for all cells to complete division is shortened to 3–4 hr.

A further demonstration of the inhibitory effect of light on cell division can be seen in Figure 3. The curves describing growth rates of low- and high-temperature strains at two levels of illuminance are plotted against temperature and are extrapolated to the lowest temperatures at which growth is barely possible. These curves are for the performance of the two strains only at lower temperatures. The complete curves for the whole temperature range are reported elsewhere.[7] For the high-temperature strain the minimal temperature for growth at 1,600 ft-c is at 20.5° and at 440 foot candles it is at 15.3°C. For the low-temperature strain the

limiting temperature for growth at 1600 ft-c is at 7.8°C and at 440 ft-c. it is at 6.6°C. There is a good reason to believe that this sensitivity of the over-all process of growth at lower temperature to light intensity is due to the adverse effects of light on cell division. If the sensitivity of cell division to high light intensity extends to higher temperatures this would account for the higher multiplication rate in the absence of cell division in synchronized algal suspensions.

It has been shown that the rates of several metabolic processes rapidly increase with the progress in cell development in Chlorella.[2,6] After reaching a peak of highest activity, the rates start to decline until in the older cells the metabolic activity becomes very low. These mature cells, even though capable of cell division

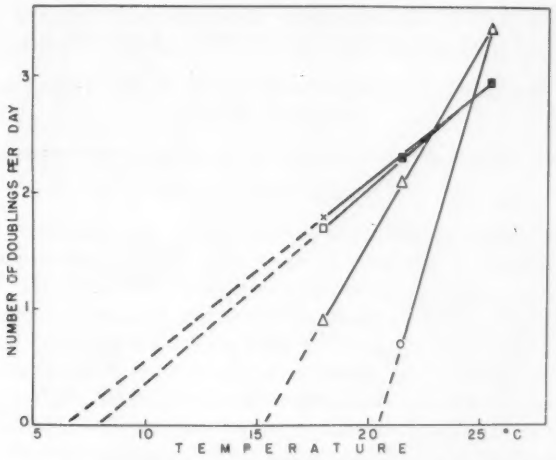

FIG. 3.—Growth rates of the high- and low-temperature strains of Chlorella extrapolated to minimal temperatures. Circles, high-temperature strain at 1,600 ft.-c; triangles, high-temperature strain at 440 ft.-c.; squares, low-temperature strain at 1,600 ft.-c.; crosses, low-temperature strain at 440 ft.-c. Solid lines are based on data obtained from measurements; broken lines are results of extrapolation of experimental data to minimal temperatures.

do not divide promptly if left in the light. Consequently their productivity is low at this stage of development. A steady-state microbial population consists of cells of all possible sizes and developmental stages. Older cells with the reduced capacity for metabolic activity and for growth decrease the total metabolic activity of the culture. The observed growth rates of such steady-state algal suspensions suffer since any characteristic obtained for such a suspension is actually a statistical average of characteristics of individual cells.

Whether there is any possibility of identifying in detail the factors which slow anabolism at the time of cell division remains a question. It appears likely that certain of the wavelengths of white light are more damaging to cell division than others which are adequate for photosynthesis. If it were indeed so then the rates of growth of the algae could be increased markedly over an extended period of time.

* This work has been supported by funds from the Office of Naval Research.
† Scientific Article A806, Contribution No. 3077 of the University of Maryland Agricultural Experiment Station.

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*PARAMAGNETIC RESONANCE SPECTRA OF FREE RADICALS TRAPPED ON IRRADIATION OF CRYSTALLINE CARBOHYDRATES**

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Communicated October 12, 1959

Employing deuterium-substituted compounds, paramagnetic (electron-spin) resonance spectroscopy was used to identify the trapped radical on irradiation of crystalline glycolic acid[1] as carboxyhydroxymethyl, $HO\dot{C}HCO_2H$. Data obtained in a paramagnetic resonance study of irradiation damage in crystalline carbohydrates exposed to ionizing radiations were reported previously[2] but allowed few structural conclusions to be drawn as to the nature of the stable free radicals formed. In continuation of this work, we have studied the electron-spin resonance spectra of other irradiated crystalline sugars and are now able to identify more closely the probable nature of some of the stable free radicals formed on the irradiation of carbohydrate materials.

Experimental.—Powdered crystalline samples of β-maltose monohydrate, β-cellobiose, α,α-trehalose, raffinose, β-D-glucuronic acid and α-D-galacturonic acid monohydrate were sealed in the presence of air in tin cans and exposed to gamma radiation in doses of 5 megarads each. The radiation was provided by the 100,000 curies fuel element gamma ray source located at the Argonne National Laboratory. This source consists of a number of fuel elements placed in a rack. The samples were placed in the rack in order to be irradiated. The irradiated samples were stored (for 6 months) in a deep freeze ($-15°$) before and throughout the investigation, but during the irradiation, the temperature was that of the source. In transit, the samples were kept at ambient-air temperature.

The spectrograph employed in this investigation consisted of an X-band Klystron operating at constant frequency (ν = 9317 Mc/sec.), controlled by a secondary frequency standard monitored by WWV. Small amounts of each of the irradiated powdered samples were placed in melting point capillary tubes (100 × 2.0 mm). The samples were mounted in a reflection quartz resonant cavity, situated between the six-inch diameter poles of an electromagnet supplying a strong magnetic field. This magnetic field could be varied by means of a clock drive attached to a potentiometer in the magnet power supply. The magnetic field modulation at 2,000

cycles/sec. was provided by means of two small coils mounted on the pole faces of the magnet. A lock-in amplifier was employed, so that the trace displayed on the recorder chart was the first derivative of the absorption signal. The magnetic field was homogeneous to within 1 gauss over the sample size used. β-D-Glucuronic acid and α-D-galacturonic acid monohydrate gave signals which were ten times stronger than those obtained from the other carbohydrates. In order to obtain more information about the nature of radicals formed from the irradiated carbohydrates of similar structure, samples of irradiated β-maltose monohydrate and irradiated β-cellobiose were thoroughly mixed (1:1 by weight) and the electron-spin resonance

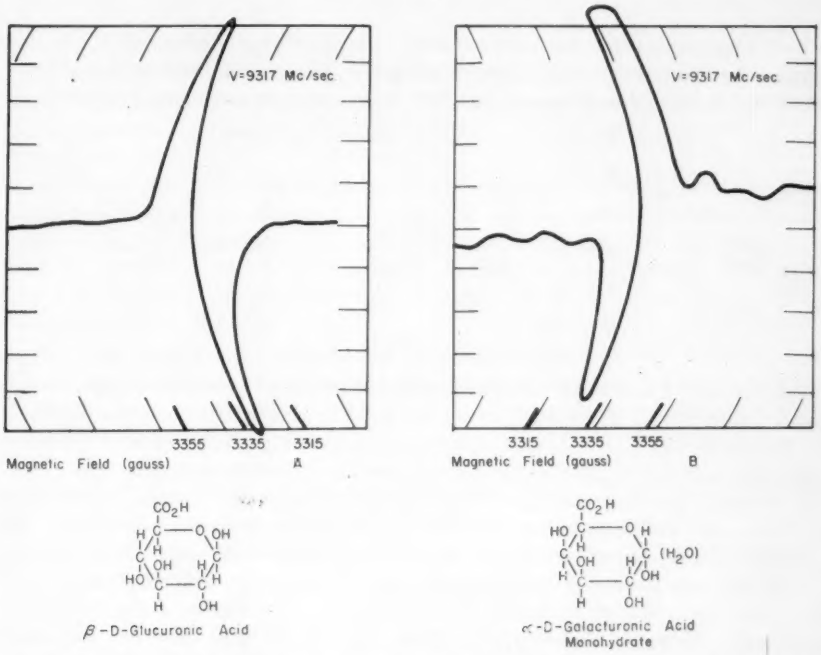


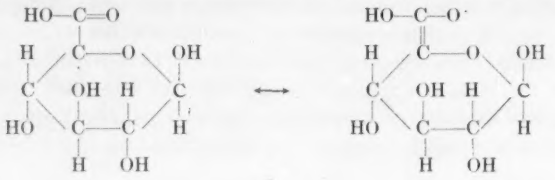
FIG. 1.—Electron spin resonance spectra of irradiated β-D-glucuronic acid and α-D-galacturonic acid monohydrate with gamma rays.

spectrum of the mixture was observed. The electron-spin resonance spectrum of a mixture (1:1 by weight) of irradiated β-D-glucuronic acid and irradiated α-D-galacturonic acid monohydrate was also observed.

The first derivatives of the absorption spectra recorded on the chart are shown in Figures 1–4.

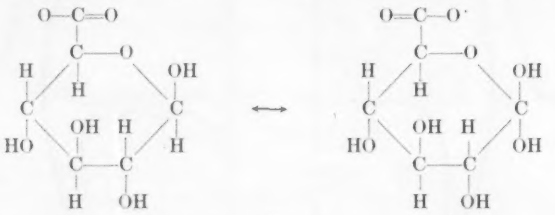
Discussion.—Irradiated β-D-glucuronic acid gave a spectrum consisting of a single peak of high intensity. The g-factor, which is calculated from the center of the spectrum, is equal to 1.996 ± 0.003 (Fig. 1, A). The width of the resonance line is 13 ± 2 gauss. Absence of an associated hyperfine structure indicates that the unpaired electron is not interacting with any neighboring hydrogen atoms. Elimination of a hydrogen atom from C5 of the D-glucuronic acid molecule would

yield a radical (I) which fits the observed spectrum. This radical could be stabilized by resonance interactions with the carboxyl group as illustrated. Another possible, but less likely radical, consistent with the observed spectrum, is that in



I

which a hydrogen atom has been removed from the carboxyl group (II). In this radical the free electron is localized on oxygen. This radical likewise should have some stabilization due to resonance. Our experiments do not allow a choice to be

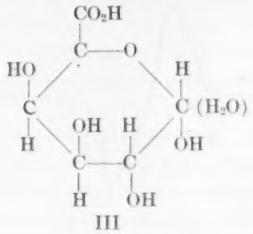


II

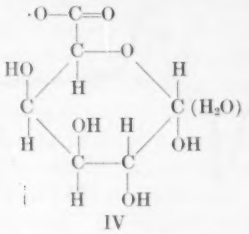
made between I and II as the stable radical observed in irradiated β-D-glucuronic acid. However, to the best of our knowledge there is no evidence for the existence of species such as II as stable, long-lived radicals, and it is believed that species of this type would very readily lose carbon dioxide. These postulations, along with the evidence for the production of the carboxyhydroxymethyl radical in irradiated glycolic acid[1] lead us to believe that I is very probably the radical observed in the electron spin-resonance spectrum of irradiated β-D-glucuronic acid. Other radicals consistent with the observed single peak would be those in which a hydrogen atom has been removed from any of the remaining carbon atoms in the D-glucuronic acid molecule. However, the simplicity of the D-glucuronic acid spectrum, compared with that obtained for irradiated D-glucose,[2] suggests that the radical observed for irradiated D-glucuronic acid is probably associated with the structural differences between the uronic acid and the simple sugar. D-Glucuronic acid differs from D-glucose only at C6, where the hydroxymethyl group of D-glucose has been replaced by a carboxyl group.

Irradiated α-D-galacturonic acid monohydrate gave a spectrum with an intense peak with a g-value equal to 1.996 ± 0.002 (Fig. 1, B). The width of the main resonance line is the same as that observed for β-D-glucuronic acid. In addition to the single intense peak, several small peaks were observed. These peaks do not appear to be part of a hyperfine structure associated with the main resonance peak, and probably indicate the presence of more than one free radical. It should be recalled that in addition to configurational differences, between D-glucuronic acid and D-galacturonic acid, the latter has associated with it one mole of water of hydration. It is suggested that the main free radical present in irradiated α-D-

galacturonic acid monohydrate is similar to that obtained for irradiated β-D-glucuronic acid. Based on the same arguments used in interpreting the spectrum obtained for D-glucuronic acid, it is suggested that III is the main radical being



III



IV

observed in irradiated α-D-galacturonic acid monohydrate. On the basis of our experimental results, IV cannot be ruled out, but we consider this radical less likely for the same reasons pointed out in discussing II as a possibility in irradiated β-D-glucuronic acid.

Additional evidence that similar radicals are formed in the irradiated uronic acids was obtained by observing the electron spin resonance spectrum of a 50 per cent mixture (by weight) of irradiated β-D-glucuronic acid and α-D-galacturonic acid monohydrate (Fig. 2, A). A single intense line was observed. This peak was very similar to the principal resonance lines of the spectrum of each of the irradiated acids.

The peak signal (Fig. 3, A) obtained from the irradiated natural trehalose (α-D-glucopyranosyl α-D-glucopyranoside) was about ten times less intense than those of the irradiated uronic acids. Two absorption peaks, which are clearly resolved, constitute the spectrum. If these peaks are associated with a hyperfine structure

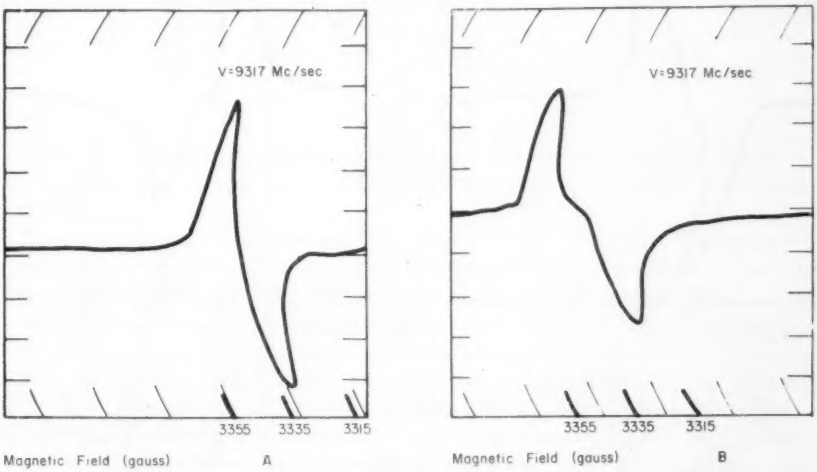


FIG. 2, A.—Electron spin resonance spectrum of a mixture (1:1 by wt.) of irradiated β-D-glucuronic acid and α-D-galacturonic acid monohydrate with gamma rays. B.—Electron spin resonance spectrum of a mixture (1:1 by wt.) of irradiated β-maltose monohydrate and irradiated β-cellobiose with gamma rays.

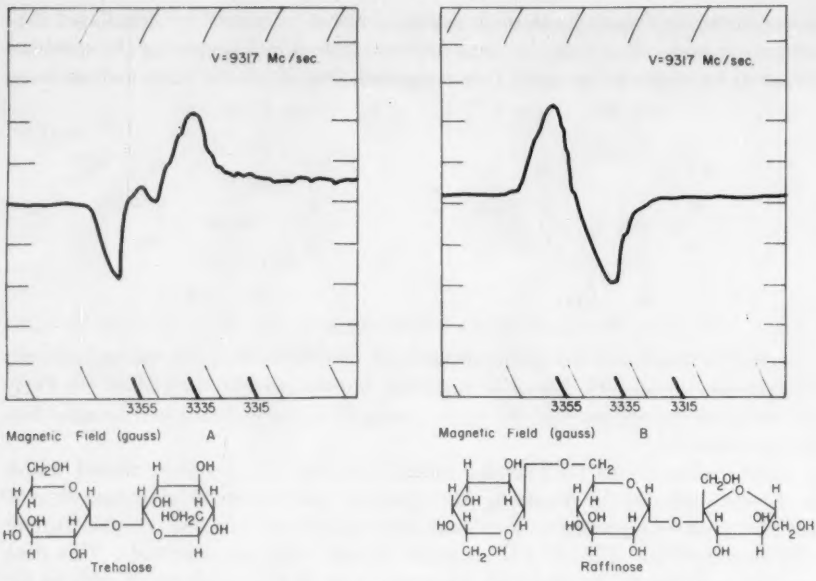


FIG. 3.—Electron spin resonance spectra of irradiated trehalose and raffinose with gamma rays.

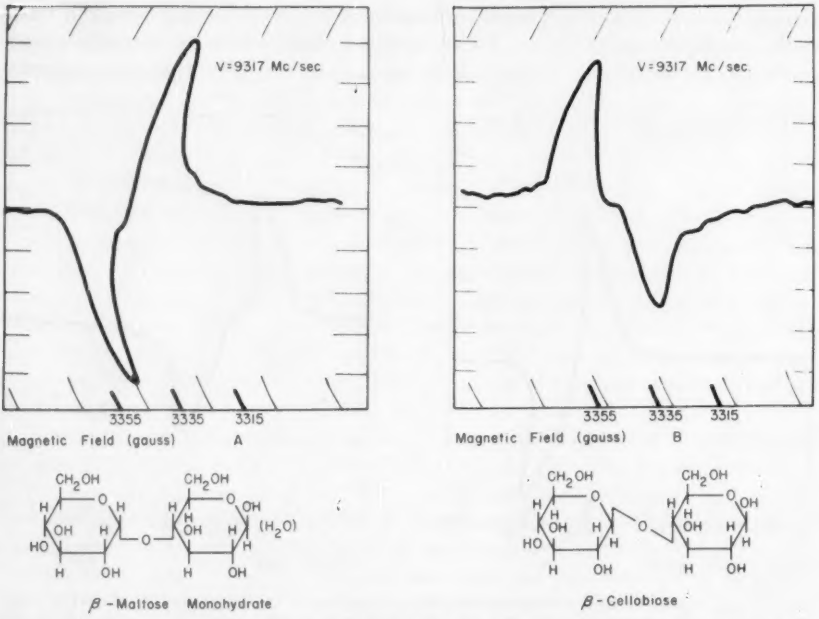


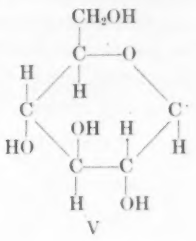
FIG. 4.—Electron spin resonance spectra of irradiated β-maltose monohydrate and β-cellobiose with gamma rays.

doublet, then, for this pair of lines ($A/g\beta$) is 10 gauss and g is 1.996 ± 0.003. This would indicate an interaction of the magnetic moment of the unpaired electron with the magnetic moment of the nearby single proton in the radical. It is probable that the unpaired electron is on a carbon atom attached to a hydrogen.

The paramagnetic resonance spectra obtained from irradiated 4-*O*-α-D-glucopyranosyl-β-D-glucose (β-maltose) monohydrate and 4-*O*-β-D-glucopyranosyl-β-D-glucose (β-cellobiose) are similar (Figs. 4, A and 4, B). In each one of them there seems to be a clear indication of splitting into a hyperfine structure doublet. This splitting is barely resolved. It is not possible to arrive at a value for ($A/g\beta$) but it appears to be less than 5 gauss. Again this would indicate interaction of the magnetic moment of the unpaired electron with that of a single proton.

The electron-spin resonance spectrum of a mixture (1:1 by weight), of irradiated maltose-irradiated cellobiose (Fig. 2, B) shows a hyperfine structure centered at $g = 1.996 \pm 0.003$. The separation of the lines is within about 7 gauss. The observed spectrum indicates that the trapped free radicals present in irradiated maltose and cellobiose are probably similar.

Hydrolysis (glycosidic cleavage) of disaccharides, as a result of irradiation in aqueous solution, has been observed in this Laboratory.[3, 4] Whether or not the observed hydrolysis is due to direct attack by irradiation, or indirectly by the attack of irradiated water fragments, has not been proved. If the observed effect is by direct action of the ionizing radiation, then a radical formed at the glycosidic linkage would be expected. The D-glucopyranosyl radical V could be formed in



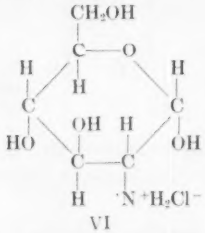
V

irradiated crystalline β-maltose monohydrate, β-cellobiose, and α,α-trehalose. This radical is consistent with the observed spectrum in each case (Figs. 3, B, 4, A and 4, B).

The electron-spin resonance spectrum obtained from irradiated *O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl β-D-fructofuranoside (raffinose), shown in Figure 3, B, is rather similar to those obtained from the irradiated disaccharides. There seems to be a slight indication of splitting into a hyperfine structure doublet. This splitting is just barely resolved. The ($A/g\beta$) appears to be less than 5 gauss. Again this would indicate interaction of the magnetic moment of the unpaired electron with that of a single proton. The structure of the trisaccharide raffinose is considerably more complex than that of the irradiated disaccharides, yet the spectrum of irradiated raffinose is similar to that observed for the irradiated disaccharides. It is suggested that, as for the disaccharides, the radical is formed at the glycosidic bonds in raffinose. It is noted that hydrolysis was observed when aqueous solutions of raffinose were irradiated.[3]

The radicals formed in all the irradiated cystalline carbohydrates are stable in air. In most postulated radicals the unpaired electron is located either on the potential carbonyl group or on the carbon atom next to the carbonyl group. This is in agreement with the observation of Rexroad and Gordy[5] that the carbonyl group, in some manner, prolongs the stability of the radicals.

The above interpretation of the electron-spin resonance spectra obtained from the gamma ray irradiated carbohydrates could be applied to the interpretation of the spectrum obtained from the X-ray irradiated 2-amino-2-deoxy-α-D-glucopyranose hydrochloride[2] (α-D-glucosamine hydrochloride). The irradiated α-D-glucosamine hydrochloride spectrum consists of five equally spaced lines separated by 18.5 gauss. One possibility is that this spectrum consists of a hyperfine structure due to the unpaired electron coupling nearly equally with two portons and a nitrogen, as suggested previously.[2] The resulting spectrum could be considered as a triplet with hyperfine components of equal intensity due to splitting by N^{14}; each component of the triplet would be further split into a triplet with hyperfine components with intensity ratios 1:2:1 produced by an interaction with two protons. If the splittings produced by N^{14} and the two protons were nearly equal, the intensity ratio of the resulting hyperfine lines would be 1:3:4:3:1. To make an estimation of the relative intensities of the five lines the first derivative record was integrated. Although the observed five lines were not completely resolved, the estimation made seemed to indicate that the ratio of the relative intensities was approximately 1:3:4:3:1 in accord with the proposed splitting mechanism. Although the nuclear magnetic moment of N^{14} is much smaller than those of the protons, nearly equal coupling might result if the electron were closer to the N^{14} nucleus than to the protons. The experimental evidence[2] in correlation with results reported in this paper would suggest that one of the stable free radicals present in the X-ray irradiated 2-amino-2-deoxy-α-D-glucopyranose hydrocloride could be VI.

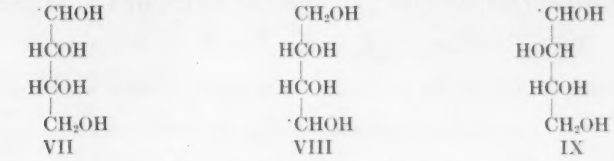


VI

Salts of similar nature have been reported in the literature[6–8] and are designated Wurster's salts. The validity of the proposed free radical VI might be checked by a study of other irradiated nitrogenous materials in the hope of determining typical values of splittings produced by N^{15}.

The spectra of irradiated erythritol and D-threitol were reported previously.[2] Both of these polyhydric alcohols gave spectra consisting of two lines of equal intensity with hyperfine spacing ($A/g\beta$) equal to approximately 6 gauss. It was indicated at that time that this type of spectrum would be expected if the electron were interacting with a single hydrogen atom in the radical. Radicals consistent with this observation could be formed by removal of a hydrogen atom from the terminal primary hydroxyl groups of erythritol and D-threitol. For erythritol

VII and VIII would be the possible radicals. For D-threitol, IX would be the likely radical, the primary hydroxyl groups being equivalent for the latter. It



is interesting to note that terminal primary hydroxyl groups are preferentially attacked when aqueous solutions of hexitols are subjected to ionizing radiation.[3]

Summary.—A number of carbohydrate substances were exposed to gamma radiation and their paramagnetic resonance spectra were studied.

The extreme simplicity of the spectra for β-D-glucuronic acid and α-D-galacturonic acid monohydrate allows a reasonable postulation to be made as to the structure of the stable free radicals present in these uronic acids.

The relative simplicity of the spectra obtained from irradiated β-maltose monohydrate, β-cellobiose, α,α-trehalose and raffinose further allows postulations to be made concerning the radicals formed on irradiation of these oligosaccharides.

On the basis of this work and published data, structures of the free radicals present in irradiated 2-amino-2-deoxy-α-D-glucopyranose hydrochloride, erythritol, and D-threitol are postulated.

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CHARACTERISTICS OF A HIGH FREQUENCY OF RECOMBINATION (HFR) STRAIN OF SALMONELLA TYPHOSA COMPATIBLE WITH SALMONELLA, SHIGELLA, AND ESCHERICHIA SPECIES

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Bacterial recombination, first demonstrated with the K-12 strains of *Escherichia coli* by Tatum and Lederberg,[1] has provided a versatile technique for the investigation of genetic organization in bacteria.[2–4] Recently, the sexual compatibility of bacteria from different genera has been extended to include hybridization between *E. coli* and Shigella,[5] and between *E. coli* and *Salmonella*.[6, 7] Only *E. coli* strains, however, have been shown to act as donors of genetic material (males) to the recipient (female) species of Escherichia, Shigella, and Salmonella. The *E. coli* male strains are of two types; the efficient donor or high frequency of recombination (Hfr) strain, and the F^+ strain, a much lower frequency mating strain which, however, has the ability to make female (F^-) strains of *E. coli* into males.[8, 9] Baron, Carey, and Spilman[7] have discussed the possibilities of developing donor strains of Salmonella previously.

It is the purpose of this communication to report on the characteristics of a *Salmonella typhosa* culture, strain ST-2, which behaves as an Hfr donor organism, compatible with many species and strains of Salmonella, Shigella, and Escherichia. Strain ST-2 is also able to confer Hfr ability on hybrids which have been selected for lactose utilization.

Materials and Methods.—Cultures: Strain ST-2 is a culture of *S. typhosa*, possessing the antigenic formula Vi 9,12:d- typical of typhoid strains. The fermentation pattern of ST-2 is like that of the well-known laboratory strain, *S. typhosa* Ty2 described by Felix and Pitt,[10] with the exception that ST-2 is able to ferment lactose and fucose. Other lactose fermenting (Lac^+) strains of *S. typhosa* obtained as a result of conjugation with *E. coli* K-12 have been reported by Baron, Spilman, and Carey.[11]

Species and strains of Shigella and Salmonella came from the culture collection of the Walter Reed Army Institute of Research. Strains of *E. coli* K-12 include W-1895 Hfr, W-1177 (F^-), and derivatives of these which will be mentioned below. These cultures, obtained from Dr. P. D. Skaar, were originally isolated by Dr. J. Lederberg and Dr. L. L. Cavalli. Other strains of K-12 were received from Dr. R. Weinberg; these were K-3 (F^-), K-152 (Hfr), and K-166 (Hfr).

Phages: The Vi typing phages described by Craigie and Felix[12] were obtained from Dr. Cora Gunther. Methods and procedures employed in propagating and studying these phages have been described by Baron, Formal, and Spilman.[13]

Media: The routine culture media as well as the methods and media used for selective purposes in recombination experiments have been described in detail.[7]

Experimental and Results.—The characteristics of strain ST-2 and its ability to recombine with Lac^- cultures are presented in Table 1. The results demonstrate that this strain of *S. typhosa* is compatible with diverse species of enteric bacteria. In some of these crosses, the frequency of recombination expressed as the ratio of

TABLE 1

CROSSES OF STRAINS WITH *Salmonella typhosa* ST-2 (HFR)
(Lac$^+$ or Lac$^+$S^r Selection)
ST-2 = Lac$^+$ Ara$^-$ Xyl$^+$ Rha$^-$ Fuc$^+$ Inos$^-$ Dul$^-$ Indol$^-$ S^s

Organism	Strain Designation	Lac$^+$ Recombinants
Salmonella typhimurium	LT-2	+
S. worthington	DC-158	+
S. paratyphi B	DC-3	+
S. typhimurium	TM-9S^r-2	+
S. norwich	DC-201	+
S. typhimurium	LT-7	+
S. champaign	WR-178	+
S. canoga	WR-244	+
S. kentucky	WR-98	+
S. illinois	WR-121	+
S. typhosa	0901	+
S. montevideo	WR-129	+
S. urbana	WR-117	+
S. typhosa	643	+
S. emek	WR-265	+
S. muenchen	WR-54	+
S. choleraesuis	DC-153	+
S. typhosa	Ty2	+
S. daytona	WR-205	+
S. florida	WR-162	+
S. lexington	WR-118	+
S. adelaide	WR-170	+
S. javiana	WR-160	
Shigella dysenteriae 2a	WR-15-1	+
Sh. flexneri 2a	WR-2457T	+
Sh. boydii 11	WR-27-2	+
Sh. flexneri 2b	WR-M58B	+
Sh. boydii 703	WR-38-1	+
Sh. flexneri 1a	WR-1-2	+
Sh. flexneri 5	WR-M90-A	+
Sh. sonnei phase II	WR-13-1	+
Sh. flexneri 4b	WR-G-2	+
Escherichia coli K-12	K-3(F$^-$Lac$-$)	+
E. coli K-12	CS-100(F$^-$Lac$-$)	+
E. coli K-12	K-166(Hfr Lac$-$)	−
E. coli K-12	K-152(Hfr Lac$-$)	−
E. coli B	B/r$_1$(F$^-$Lac$-$)	+
Paracolobactrum ballerup	WR-107	+

TABLE 2

FREQUENCY OF RECOMBINATION WITH HFR STRAINS

Recipient Strain	Donor Strains	
	E. coli W-1895	S. typhosa ST-2
Salmonella typhimurium TM-9	4×10^{-8}	2×10^{-3}
S. typhimurium TM-9S^r-2	1×10^{-4}	3×10^{-3}
S. typhimurium TM-10	0	2×10^{-3}
S. typhimurium LT-2	0	6×10^{-4}
S. typhimurium LT-7	1×10^{-7}	4×10^{-4}
S. typhosa 643	$<10^{-8}$	5×10^{-4}
Shigella flexneri 2a	2×10^{-4}	4×10^{-4}
Escherichia coli B/r$_1$ (Lac$^-$)	..	2×10^{-3}

recombinants to the number of ST-2 cells (Table 2) was approximately 2×10^{-3}, which is somewhat higher than the results reported[7] in matings of *E. coli* Hfr $\times$ *S. typhimurium* F$^-$.

After purification from these crosses, Lac$^+$ hybrids were tested for acquisition of the ability to mate as males. This was accomplished by backcrossing the hybrids with Lac$^-$ strains on minimal lactose (ML) agar in the case of Lac$^+$ hybrids of *Shigella flexneri* which are unable to grow without nicotinic and aspartic acid

(nic$^-$ asp$^-$). Lac$^+$ hybrids of nutritionally independent strains (*S. typhimurium* and other Salmonella species) were crossed with Lac$^-$ S^r strains on Eosin-Methylene Blue (EMB) agar containing streptomycin (600 micrograms/ml). These efficient selective mechanisms were used to eliminate the male parent, since hybrids result from genetic transfer to the female. Therefore, hybrids will be referred to by the name of the female parent. The results in Table 3 show that these Lac$^+$ hybrids

TABLE 3

DEMONSTRATION OF HFR ABILITY BY HYBRIDS OF CROSSES WITH *S. typhosa* ST-2 (Selection for Lac$^+$ Hybrids)

Recipient Strain	% HFR Hybrids
Salmonella typhimurium TM-9	100
S. typhimurium LT-2	100
S. typhosa Ty2	100

Mating Procedure		HFR Hybrids (Lac$^+$)
S. typhosa ST-2 Hfr Lac$^+$	× *S. typhimurium* LT-2 Lac$^-$	+
S. typhimurium LT-2 Hfr Lac$^+$	× *S. typhimurium* TM-9 Lac$^-$	+
S. typhimurium TM-9 Hfr Lac$^+$	× *S. typhosa* Ty2 Lac$^-$	+
S. typhimurium LT-2 Hfr Lac$^+$	× *Sh. flexneri* 2a Lac$^-$	+
S. typhosa Ty2 Hfr Lac$^+$	× *Sh. flexneri* 2a Lac$^-$	+
Sh. flexneri 2a Hfr Lac$^+$	× *S. typhimurium* TM-9 Lac$^-$	+
Sh. flexneri 2a Hfr Lac$^+$	× *S. typhosa* Ty2 Lac$^-$	+
Sh. flexneri 2a Hfr Lac$^+$	× *S. flexneri* 2a Lac$^-$	−
S. typhosa ST-2 Hfr Lac$^+$	× *E. coli* B/r$_1$ Lac$^-$	+

have acquired the ability to mate as male strains as a consequence of their recombination with ST-2 and indicate the crosses which have been accomplished.

The following experiment was performed in order to determine whether ST-2 behaved in these crosses as a typical Hfr strain, as the recombination frequencies observed appeared to indicate, or as an F$^+$ strain exhibiting a frequency more usually ascribed to Hfr strains. This is particularly pertinent since the F$^+$ character can be transferred to an F$^-$ strain by contact alone with the F$^+$ strain without the occurrence of genetic recombination.[8, 9] A Lac$^+$ hybrid of *S. typhimurium* strain TM-9, was obtained as an F^0 (sterile) to F$^-$ mutant[14] from a cross with *E. coli* Hfr strain W1895. This hybrid, which was unable to act as a donor, was mixed in nutrient broth with ST-2. Both organisms were grown together at 37° C and at intervals the mixed culture was streaked on ML agar plates, as a selective device to recover the TM-9 Lac$^+$ organisms. Growth of ST-2 is not supported by this medium, since it has a nutritional requirement for cystine and tryptophan (cys$^-$ try$^-$). Attempts made in this manner to detect transfer of the F$^+$ character by contact were not successful, as judged by the lack of male sexual potency of these TM-9 Lac$^+$ isolates in subsequent crosses. Lac$^+$ TM-9 hybrids, which are fully competent as Hfr males, however, can be obtained following genetic recombination with TM-9 as the recipient strain and ST-2 as donor strain (100 per cent of these hybrids are Hfr after selection for Lac$^+$). Similar experiments using *E. coli* B also failed to show an acquisition of mating ability solely through contact with ST-2.

Strain ST-2 was next examined for its possible behavior as a female or recipient strain, as were the Lac$^+$ *S. typhosa* hybrids previously studied by Baron, Spilman, and Carey.[11] For this purpose, strain ST-2 was grown on nutrient agar plates, rather than in nutrient broth, harvested, and washed thoroughly; this method of cultivation results in a phenotypic change of donor cultures of *E. coli* from the male

to the female state.[15] Loss of donor ability was noted with ST-2 grown in this way. ST-2 was then mated with the *E. coli* Hfr strain, W-1895, on minimal media supplemented with cystine and tryptophan with either l-arabinose or l-rhamnose as the sole carbon source. Strain ST-2 was found to react as a recipient (phenotypically F^-) strain in these experiments with frequencies similar to those reported for other F^- *S. typhosa* previously studied.[11, 14] Thus, in this manner, it was possible to obtain hybrids of ST-2 which are able to utilize l-arabinose, and/or l-rhamnose, and which can produce indol. These hybrids still retained their ability to mate as males with the cultures listed in Table 1. A number of highly unstable forms, probably similar to the diploids described by Lederberg,[16] and a more stable hybrid which cross-reacted with antiserum to *E. coli*, identical in most aspects to a piliated hybrid of *S. typhosa*[17] previously studied, were observed and isolated in these experiments.

S. typhosa ST-2 was tested for lytic pattern with the Vi phage II typing phages. The results established this strain as a degraded Vi type, i.e. a strain lysed by all of the typing phages. All of the hybrids of ST-2 tested were still Vi^+ and susceptible to the Vi phages, including those hybrids which cross-reacted serologically with the *E. coli* donor strain.

The Lac^+ *Shigella flexneri* hybrids obtained from crosses with *S. typhosa* ST-2 were found to have retained their specific antigens in contrast to the majority of those isolated by Luria and Burrous[5] as a result of matings between *E. coli* and Shigella.

The selective marker employed throughout these mating experiments was the ability to utilize lactose (Lac^+). No instance of a spontaneous mutation to Lac^+ was observed with any of the Salmonella or Shigella cultures tested in these crosses; reversion of some Lac^- Escherichia strains to Lac^+ was observed on control plates, but recombination frequencies for Lac^+ hybrids of Lac^- *E. coli* were far in excess of the reversion frequencies. Since *S. typhosa* ST-2 is unable to utilize inositol, arabinose, or rhamnose, and cannot produce indol, loss of some of these abilities as unselected markers was tested for and detected in some Lac^+ hybrids. These results will be reported in a detailed communication at a later date.

The extension of compatibility with *E. coli* strains to Salmonella species was interpreted to be the result of F^- mutations in an otherwise F^0 (sterile) population of Salmonella cells.[14] The results now indicate clearly that Salmonella species which are F^0 in *E. coli* matings can, nevertheless, be fertile as recipients (F^-) at high frequency with *S. typhosa* ST-2. In addition, strains of *E. coli* K-12, Shigella, or Salmonella which behave as typical F^- cultures in crosses with *E. coli* show no fertility advantage over F^0 species of Salmonella, in crosses with ST-2. It was observed, however, that a Salmonella strain (*S. typhosa* Ty2) F^0 with regard to its mating ability with *E. coli* Hfr strain W1895, was able to mate with this strain of *E. coli*, after hybridization with ST-2. This experiment is depicted in Table 4, where it can be seen that the Lac^+ Hfr hybrid of *S. typhosa* Ty2 has acquired the ability to act as a recipient strain (female) in a cross with the *E. coli* Hfr.

Discussion.—The results of this preliminary survey of the mating capabilities of *S. typhosa* strain ST-2 indicate that this organism behaves as a male strain. Furthermore, it appears to act as a typical Hfr culture, as determined by the high frequency at which recombinants are detected as well as by its inability to transfer

TABLE 4

ACQUISITION OF FERTILITY WITH *E. coli* HFR BY A HYBRID FROM A CROSS WITH *S. typhosa* ST-2

Crosses	Frequency of Recombination Selection for LAC+	Selection for *l*-ARA+
1. *E. coli* Hfr × *S. typhosa* Ty2(F^0)	$< 10^{-8}$	0
2. *S. typhosa* ST-2 Hfr × *S. typhosa* Ty2	2×10^{-4}	0
3. *E. coli* Hfr × *S. typhosa* Ty2(Lac+ Hfr)*	..	3×10^{-7}
4. *E. coli* Hfr × *S. typhosa* 643(F^0)	$< 10^{-8}$	0
5. *E. coli* Hfr × *S. typhosa* 643(Lac+ F−)†	..	2×10^{-6}

* Hybrid from cross 2 increase in frequency due presumably to mating with ST-2.
† Hybrid from cross 4 increase in frequency due to selection of F^0 to F− mutant.

the fertility factor (F) by contact as would be expected of an F+ culture. It is, nevertheless, able to transfer the Hfr character to recipient strains or species of Salmonella, Shigella, or Escherichia as a consequence of hybridization and these hybrids are now able to act as donor strains in subsequent matings, usually with the same high frequency of recombination as the original ST-2.

It is felt that this discovery may offer many unique opportunities and advantages for further genetic exploration. Heretofore, hybridization had been limited to certain strains of certain species with only *E. coli* strains capable of performing as Hfr or F+ males. Now, Hfr strains of Salmonella, Shigella, and Escherichia can be derived easily, and in fact, occur in all hybrids selected for ability to utilize lactose (Lac+) which have been tested. *S. typhosa* ST-2, and other Hfr strains of Salmonella, Shigella, and Escherichia which have been developed, still are able to act as F− strains under the proper conditions. Thus, it is possible to introduce other markers into these strains by appropriate crosses without affecting their Hfr ability (in instances examined to date).

The observation that all the Salmonella, Shigella, and Escherichia cultures that have been tested with *S. typhosa* ST-2 were fertile, regardless of whether or not they were classified as F− in matings with *E. coli*, is especially noteworthy. It now seems that all enteric strains studied can be fertile in certain crosses, though sterile in other matings, while the typical F− strains previously described would be fertile in all crosses. This situation is pertinent to the present compatibility scheme, as preliminary findings demonstrate that an F^0 strain in an *E. coli* mating can be made fertile for this same *E. coli* donor, after hybridization with ST-2. Although the fertility here was at a lower frequency than that observable in the case of a typical F− (one selected as an F^0 to F− mutation by mating with *E. coli*[14]), this enhancement of fertility would be of obvious importance were it a general phenomenon.

The invariable transfer of efficient donor ability to Lac+ hybrids indicates that this factor (Hfr) is linked to Lac in ST-2. Further studies will be necessary to determine the order of entry of markers in these crosses, although the Hfr linkage to Lac and other preliminary evidence demonstrate a marked difference in behavior of strain ST-2 as compared to the *E. coli* Hfr strains previously studied in this laboratory.

The alterations of the genomes of the enteric species which have been described may allow the creation of hybrids of novel biochemical and antigenic characteristics. Such changes may perhaps establish a rational basis for the difference in the pathogenicity of bacterial strains. This problem has been admirably presented by Burnet[18] in a discussion on speculations from a medical angle.

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[16] Lederberg, J., these PROCEEDINGS, **35**, 178 (1949).

[17] Brinton, C. C., Jr., and L. S. Baron, to be published.

[18] Burnet, F. M., *Cold Spring Harbor Symposia Quant. Biol.*, **23**, 1 (1958).

The following is quoted from Dr. Burnet's article:

"Virulence is an inheritable character—from the point of view of medicine it is the most important character of all, and as such worthy of close genetic study. I am rather sorry that, for understandable reasons, very little refined genetic study has yet been made of the phenomena of bacterial virulence. It might be a happy thought if someone switched from *E. coli* B and K-12 to pathogenic strains of *S. pullorum* or *gallinarum* which can readily be tested for pathogenicity. . . ."

ON FINITE GROUPS OF EVEN ORDER WHOSE 2-SYLOW GROUP IS A QUATERNION GROUP

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We shall sketch a proof of the following theorem:[1]

THEOREM 1. *Let G be a group of finite even order. If the 2-Sylow group P of G is a quaternion group (ordinary or generalized), then G is not simple.*

Proof: If P has order 2^n, it can be generated by two elements α and β with

$$\alpha^{2^{n-1}} = 1, \qquad \beta^2 = \alpha^{2^{n-2}}, \qquad \beta^{-1}\alpha\beta = \alpha^{-1}.$$

The element $\mu = \alpha^{2^{n-2}}$ is the only element of order 2 in P and hence all elements of order 2 in G are conjugate to μ in G. Let $\chi_1 = 1, \chi_2, \ldots, \chi_k$ denote the irreducible characters of G and set $x_i = \chi_i(1)$. If σ is an element of G of even order, it can be shown without difficulty[2] that

$$\sum_{i=1}^{k} \chi_i(\mu)^2 \chi_i(\sigma)/x_i = 0. \tag{1}$$

Let $\pi \neq 1$ be a fixed element of P and let ρ be an element of odd order of the

centralizer $C(\pi)$ of π. Applying the theory of modular character for the prime $p = 2$, we have

$$\chi_i(\pi\rho) = \sum_j d_{ij}^{\pi}\varphi_j^{\pi}(\rho) \tag{2}$$

where the d_{ij}^{π} are the decomposition numbers of G for $p = 2$ and where $\varphi_1^{\pi} = 1$, φ_2^{π}, φ_3^{π}, ... are the irreducible modular characters of $C(\pi)$ for $p = 2$. On substituting (2) in (1) for $\sigma = \pi\rho$ and using the linear independence of the φ_j^{π}, we obtain

$$\sum_i \chi_i(\mu)^2 d_{ij}^{\pi}/x_i = 0, \ (j = 1,2,\ldots). \tag{3}$$

Assume first that P is a generalized quaternion group, $n > 3$. We let π range over the elements of $\{\alpha\}$ of orders 2^{n-1} and 2^{n-2}. It can be seen that there exists a linear combination of the corresponding equations (3) with $j = 1$ of the form

$$\sum_i \chi_i(\mu)^2 t_i/x_i = 0 \tag{4}$$

such that the t_i are rational integers, $t_i = 1$, and

$$\sum_i t_i^2 = 3. \tag{5}$$

It follows from the properties of the decomposition[3] numbers that

$$(a) \quad \sum_i t_i x_i = 0; \quad (b) \quad \sum_i t_i\chi_i(\mu) = 0. \tag{6}$$

Because of (5), only three of the t_i are different from 0. On combining (4) and (6), we can deduce $\chi_i(\mu) = \chi_i(1)$ for these three values of i. Hence μ belongs to the kernels of the corresponding representations and G cannot be simple.

If P is the ordinary quaternion group, the argument is more complicated. We may assume that G does not have a normal subgroup of index 2. By the *principal block* of characters of a group, we mean the block which contains the principal character $\chi_1 = 1$ (for the given prime p, here $p = 2$). We take $\pi = \alpha$ and $\pi = \mu$ choosing for φ_j^{π} the irreducible modular characters of the principal block of $C(\pi)$. For $\pi = \alpha$, we have only $\varphi_1^{\mu} = 1$ while for $\pi = \mu$ we have three characters φ_j^{μ}. This gives us four equations (3). In addition, we use (2) and the properties of the decomposition numbers, in particular, the orthogonality relations.[3] If the characters χ_i are taken in a suitable order, it can be shown that there exist signs $\delta_1 = 1$, $\delta_2 = \pm 1, \delta_3 = \pm 1, \ldots$ such that the following relations hold:

(*a*) The equations (4) and (6) with $t_i = \delta_i$ for $1 \leqq i \leqq 4$ and $t_i = 0$ for $i > 4$.

(*b*) The equations (4) and (6a) in the following three cases:

1) $t_1 = \delta_1, t_4 = -\delta_4, t_6 = \delta_6, t_7 = \delta_7$, all other $t_i = 0$;
2) $t_2 = \delta_2, t_4 = -\delta_4, t_5 = \delta_5, t_7 = \delta_7$, all other $t_i = 0$,
3) $t_3 = \delta_3, t_4 = -\delta_4, t_5 = \delta_5, t_6 = \delta_6$, all other $t_i = 0$;

(*c*) $\delta_5\chi_5(\mu) = \delta_2\chi_2(\mu) + \delta_3\chi_3(\mu)$, $\delta_6\chi_6(\mu) = \delta_1\chi_1(\mu) + \delta_3\chi_3(\mu)$, $\delta_7\chi_7(\mu) = \delta_1\chi_1(\mu) + \delta_2\chi_2(\mu)$.

An elementary, but somewhat messy, computation allows to deduce $\chi_2(\mu) = x_2$. Again, G cannot be simple.

A more detailed discussion of the decomposition numbers leads to the following refinement of Theorem 1:

THEOREM 2. *Let G be a finite group whose 2-Sylow group is a quaternion group (ordinary or generalized). If H is the unique maximal normal subgroup of odd order, then G/H has a center of order 2.*

An example is given by the extended icosahedral group of order 120.

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† The work of the second author was supported by a Faculty Summer Fellowship from the University of Illinois.

[1] Theorem 1 in the case that P is the ordinary quaternion group was obtained by R. Brauer and communicated to M. Suzuki who then also gave a proof. Both authors independently obtained the extension to the case that P is a generalized quaternion group.

[2] Cf. Brauer, R., and K. A. Fowler, *Annals of Mathematics*, **62**, 565–583 (1955), in particular, Theorem (2A) and equation (23).

[3] For the results used here, cf. Brauer, R., *Annals of Mathematics*, **42**, 926–935 (1941); these PROCEEDINGS, **32**, 215–219 (1946).

GENERALIZED SPACES OF GENERAL RELATIVITY

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1. This paper deals with a space of four dimensions V_4 of coordinates x^i as i takes the values 1 to 4. The geometry of the space is based in part on a set of asymmetric coefficients of connection Γ^i_{jk} in coordinates x^i which are related to the coefficients $\Gamma'^{\delta}_{\alpha\beta}$ in the coordinates x'^{α} by the equations

$$x^i_{,\alpha\beta} + \Gamma^i_{jk} x^j_{,\alpha} x^k_{,\beta} = \Gamma'^{j}_{\alpha\beta} x^i_{,\gamma} \tag{1}$$

Throughout this paper a quantity followed by a comma and an index denotes the derivatives of the quantity with respect to x or x' with this index as the case may be. Also when there is a repeated upper and lower index, the one term stands for the sum of terms as this index takes the values 1 to 4. Thus in equations (1) the second term stands for the sum of terms as j and k take the values 1 to 4, and in the right-hand member of the equation as γ takes the values 1 to 4.

When we express the condition of integrability of equations (1) and make use of these equations in the reduction, we obtain[1]

$$\Gamma^i_{jkl} x^j_{,\alpha} x^k_{,\beta} x^l_{,\gamma} = \Gamma'^{\sigma}_{\alpha\beta\gamma} x^i_{,\sigma}$$

where

$$\Gamma^i_{jkl} = \Gamma^i_{jk,l} - \Gamma^i_{jl,k} + \Gamma^m_{kl}\Gamma^i_{jk} - \Gamma^i_{hk}\Gamma^h_{jl} \tag{2}$$

and similarly for $\Gamma'^{\sigma}_{\alpha\beta\gamma}$. Hence Γ^i_{jkl} and $\Gamma'^{\sigma}_{\alpha\beta\gamma}$ are components of a tensor.

When in equation (2) the functions of the form Γ^i_{jk} are replaced by the Christoffel

symbols $\left\{ {i \atop j\ k} \right\}$ in terms of the symmetric g_{ij} of Riemannian space, the tensor Γ^i_{jkl} becomes the Riemann curvature tensor R^i_{jkl}.[2]

2. The covariant derivatives with respect to x^j of the vectors λ_i and λ^i denoted by $\lambda_{i;j}$ and $\lambda^i_{;j}$ are defined by

$$\lambda_{i;j} = \lambda_{i,j} - \lambda_h \Gamma^h_{ij}, \quad \lambda^i_{;j} = \lambda^i_{,j} + \lambda^h \Gamma^i_{hj} \tag{3}$$

It is readily proved by means of equations of the form (1) that $\lambda_{i;j}$ and $\lambda^i_{;j}$ are components of tensors.

For a nonsymmetric invariant tensor a_{ij} the covariant derivative with respect to x^k denoted by $a_{ij;k}$ is defined by[3]

$$a_{ij;k} = a_{ij,k} - a_{ih}\Gamma^h_{kj} - a_{hj}\Gamma^h_{ik} \tag{4}$$

That $a_{ij;k}$ are the components of a tensor is established by means of equations of the form (1).

Following Einstein, we introduce the fundamental nonsymmetric tensor g_{ij} and require that the functions Γ^h_{ij} be such that the equations

$$g_{ij;k} = g_{ij,k} - g_{ih}\Gamma^h_{kj} - g_{hj}\Gamma^h_{ik} = 0 \tag{5}$$

be satisfied.[4] Determination of the expressions of the Γ^h_{ij} in terms of the derivatives of the g_{ij} is made in the next section.

The expressions for the quantities Γ^h_{ij} as derived in the next section involve components of the contravariant nonsymmetric tensor g^{ij} related to the g_{ij} by equations of the form

$$g^{hi}g_{hj} = \delta^i_j \tag{6}$$

where δ^i_j is $+1$ or zero according as i and j are the same or different numbers.

When equation (6) is differentiated with respect to x^k and the quantity $g_{hj,k}$ is replaced by its expression from an equation of the form (5), the result with change of certain repeated indices is

$$\Gamma^i_{kj} + g_{ij}g^{hi}\Gamma^l_{hk} + g_{ij}g^{li}{}_{,k} = 0$$

When the first term in this equation is replaced by $g^{lh}g_{ij}\Gamma^i_{kh}$ summed for h and l, the equation may be written

$$g_{ij}(g^{li}{}_{,k} + g^{hi}\Gamma^l_{hk} + g^{lh}\Gamma^i_{kh}) = 0$$

Since this equation holds for each value of j, it follows that the quantity in parenthesis is equal to zero, that is with change of indices

$$g^{ij}{}_{;k} = g^{ij}{}_{,k} + g^{hj}\Gamma^i_{hk} + g^{ih}\Gamma^j_{kh} = 0 \tag{7}$$

2. Write equation (5) in the form

$$g_{\underline{ij},k} + g_{\underset{\sim}{ij},k} = (g_{\underline{ih}} + g_{\underset{\sim}{ih}})(\Gamma^h_{\underline{jk}} - \Gamma^h_{\underset{\sim}{jk}}) + (g_{\underline{jh}} - g_{\underset{\sim}{jh}})(\Gamma^h_{\underline{ik}} + \Gamma^h_{\underset{\sim}{ik}})$$

where $g_{\underline{ij}}$ and $g_{\underset{\sim}{ij}}$ are the symmetric and skew-symmetric parts of g_{ij}, and $\Gamma^h_{\underline{jk}}$ and $\Gamma^h_{\underset{\sim}{jk}}$ are the symmetric and skew symmetric parts of Γ^h_{jk}. This type of notation is used throughout this paper. When i and j are interchanged in this equation, one has

$$g_{\underline{ij},k} - g_{\underset{\sim}{ij},k} = (g_{\underline{ih}} - g_{\underset{\sim}{ih}})(\Gamma^h_{\underline{jk}} + \Gamma^h_{\underset{\sim}{jk}}) + (g_{\underline{jh}} + g_{\underset{\sim}{jh}})(\Gamma^h_{\underline{ik}} - \Gamma^h_{\underset{\sim}{ik}})$$

One-half the sum and one-half the difference of these two equations are the equations

$$g_{\underline{ij},k} = g_{\underline{ih}}\Gamma_{\underline{jk}} + g_{\underline{jh}}\Gamma^{h}_{\underline{ik}} - g_{\underline{ih}}\Gamma^{h}_{\underline{jk}} - g_{\underline{jh}}\Gamma_{\underline{ik}}, \tag{8}$$

$$g_{\underline{ij},k} = g_{\underline{ih}}\Gamma^{h}_{\underline{jk}} - g_{\underline{jh}}\Gamma^{h}_{\underline{ik}} - g_{\underline{ih}}\Gamma^{h}_{\underline{jk}} + g_{\underline{jh}}\Gamma^{h}_{\underline{ik}} \tag{9}$$

We impose upon the skew-symmetric Γ's conditions of the type

$$g_{\underline{ih}}\Gamma^{h}_{\underline{jk}} + g_{\underline{jh}}\Gamma^{h}_{\underline{ik}} = 0 \tag{10}$$

and equation (8) becomes

$$g_{\underline{ij},k} = g_{\underline{ih}}\Gamma^{h}_{\underline{jk}} + g_{\underline{jh}}\Gamma^{h}_{\underline{ik}} \tag{11}$$

From equations of this form the equation

$$g_{\underline{ik},j} + g_{\underline{jk},i} - g_{\underline{ij},k} = 2g_{\underline{hk}}\Gamma^{h}_{\underline{ij}} \tag{12}$$

is derived.

Equation (10) is satisfied identically by

$$\begin{aligned} g_{\underline{ih}}\Gamma^{h}_{\underline{jk}} &= {}^1/_2\,(g_{\underline{jk},i} - g_{\underline{ik},j} + g_{\underline{ij},k}) \\ g_{\underline{jh}}\Gamma^{h}_{\underline{ik}} &= {}^1/_2\,(g_{\underline{ik},j} - g_{\underline{jk},i} + g_{\underline{ij},k}) \end{aligned} \tag{13}$$

3. Proceeding with equation (7) as was done with equation (5) resulting with equations (8) and (9) one obtains

$$\begin{aligned} g^{\underline{ij}}{}_{,k} &= g^{\underline{hj}}\Gamma^{\underline{i}}_{\underline{hk}} + g^{\underline{hi}}\Gamma^{\underline{j}}_{\underline{hk}} - g^{\underline{hj}}\Gamma^{\underline{i}}_{\underline{hk}} - g^{\underline{hi}}\Gamma^{\underline{j}}_{\underline{hk}} \\ g^{\underline{ij}}{}_{,k} &= -g^{\underline{hj}}\Gamma^{\underline{i}}_{\underline{hk}} + g^{\underline{hi}}\Gamma^{\underline{j}}_{\underline{hk}} - g^{\underline{hj}}\Gamma^{\underline{i}}_{\underline{hk}} + g^{\underline{hi}}\Gamma^{\underline{j}}_{\underline{hk}} \end{aligned} \tag{14}$$

For

$$g^{\underline{hi}}g_{\underline{hj}} = \delta^{i}_{j}, \qquad g^{\underline{hi}}g_{\underline{hj}} = \delta^{i}_{j} \tag{15}$$

equation (6) becomes

$$g^{\underline{hi}}g_{\underline{hj}} + g^{\underline{hi}}g_{\underline{hj}} = -\delta^{i}_{j} \tag{16}$$

When equation (10) is multiplied by $g^{\underline{ij}}$ and summed for i and j, the result is

$$\Gamma^{j}_{\underline{jk}} + \Gamma^{i}_{\underline{ik}} = 0$$

Since similar results follow for all equations of the type (10), it follows that

$$\Gamma^{i}_{\underline{ik}} = g^{\underline{ij}}g_{\underline{ki},j} = 0 \tag{17}$$

for all values of k.

When the expressions for $g^{\underline{ij}}{}_{,k}$ from (14) and $g_{\underline{il},k}$ from (11) are substituted in the equation

$$(g^{\underline{ij}}g_{\underline{il}})_{,k} = g^{\underline{ij}}{}_{,k}g_{\underline{il}} + g^{\underline{ij}}g_{\underline{il},k} = 0$$

the various symmetric $\Gamma^{h}_{\underline{ij}}$ cancel out and the result is

$$g_{\underline{il}}(g^{\underline{hj}}\Gamma^{\underline{i}}_{\underline{hk}} + g^{\underline{hi}}\Gamma^{\underline{j}}_{\underline{hk}}) = 0$$

Since this result holds for all values of l, one has

$$g^{\underline{hj}}\Gamma^{\underline{i}}_{\underline{hk}} + g^{\underline{hi}}\Gamma^{\underline{j}}_{\underline{hk}} = 0 \tag{18}$$

As a result of this equation the first of equations (14) becomes

$$g^{\underline{ij}}{}_{,k} = -g^{\underline{hj}}\Gamma^{\underline{i}}_{\underline{hk}} - g^{\underline{hi}}\Gamma^{\underline{j}}_{\underline{hk}} \tag{19}$$

When the expressions for $g^{\underline{ij}}{}_{,k}$ and $g_{\underline{il},k}$ from (14) and (9) are substituted in the equation

$$(g^{\underline{ij}}g_{\underline{il}})_{,k} = g^{\underline{ij}}{}_{,k}g_{\underline{il}} + g^{\underline{ij}}g_{\underline{il},k} = 0$$

the various symmetric $\Gamma^{\underline{h}}_{\underline{ij}}$ cancel out and the result is

$$g_{\underline{il}}(g^{\underline{hj}}\Gamma^{\underline{i}}_{\underline{hk}} - g^{\underline{hi}}\Gamma^{\underline{j}}_{\underline{hk}}) + g^{\underline{ij}}(g_{\underline{ih}}\Gamma^{\underline{h}}_{\underline{lk}} - g_{\underline{lh}}\Gamma^{\underline{h}}_{\underline{ik}}) = 0 \tag{20}$$

By means of equations of the type (16) this equation is given the form

$$g^{\underline{hj}}(g_{\underline{il}}\Gamma^{\underline{i}}_{\underline{hk}} + g_{\underline{hi}}\Gamma^{\underline{i}}_{\underline{lk}}) - g_{\underline{lh}}(g^{\underline{hi}}\Gamma^{\underline{j}}_{\underline{ik}} + g^{\underline{ji}}\Gamma^{\underline{h}}_{\underline{ik}}) = 0$$

When the expressions for the Γ's from (13) are substituted, the equation is satisfied identically. This means that equations (20) do not impose conditions on the first derivatives of the $g_{\underline{ij}}$.

4. When the equation

$$g^{\underline{ij}}g_{\underline{il}} + g^{\underline{ij}}g_{\underline{il}} = -\delta^j_l$$

from (16) is differentiated with respect to x^k and the expressions for $g^{\underline{ij}}{}_{,k}$ and $g^{\underline{ij}}{}_{,k}$ from (19) and (14) and the expressions for $g_{\underline{il},k}$ and $g_{\underline{il},k}$ from (9) and (11) with j replaced by l are substituted, the terms of the symmetric Γ's of the type $\Gamma^{\underline{h}}_{\underline{ij}}$ and of the skew-symmetric Γ's of the type $\Gamma^{\underline{h}}_{\underline{ij}}$ cancel out by means of equations of the types (15) and (16).

In this case and the preceding ones no conditions are imposed on the symmetric Γ's which means that for the generalized spaces under consideration no conditions are imposed on the first derivatives of the symmetric $g_{\underline{ij}}$. And as established for equations (20), conditions are not imposed on the first derivatives of the skew-symmetric $g_{\underline{ij}}$. This result is the purpose of the paper.

[1] Eisenhart, "Non-Riemannian Geometry," *Amer. Math. Soc. Pubs.* **8** (1927), p. 4.

[2] Eisenhart, *Riemannian Geometry* (Princeton: Princeton University Press, 1949), p. 19.

[3] Eisenhart, "Non-Riemannian Geometry," p. 6.

[4] Einstein, *The Meaning of Relativity* (Princeton: Princeton University Press, 1953), 4th ed., p. 134.

SOME RESULTS ON DIFFERENTIAL EQUATIONS AND THEIR APPLICATIONS*

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Let $\mathbf{R}$ and $\mathbf{C}$ be the fields of real and complex numbers respectively and $\mathbf{Z}_+$ the set of all nonnegative integers. Fix an integer $l \geq 1$ and denote by $\mathbf{C}^l$ the Cartesian product of $\mathbf{C}$ with itself l times. Similarly for $\mathbf{R}^l$ and $\mathbf{Z}_+{}^l$. Put $\langle \mathbf{t}, \mathbf{t}' \rangle = t_1 t_1' + \ldots + t_l t_l'$ for $\mathbf{t} = (t_1, \ldots, t_l), \mathbf{t}' = (t_1', \ldots, t_l')$ in $\mathbf{C}^l$. Also let[1] $s(\mathbf{t}) = \mathrm{Re}(t_1 + \ldots + t_l)$, $|\mathbf{t}| = \sup_j |t_j|$ and $\mathbf{t}^{\mathbf{k}} = t_1^{k_1} t_2^{k_2} \ldots t_l^{k_l}$ $(\mathbf{t} \in \mathbf{C}^l, \mathbf{k} \in \mathbf{Z}_+{}^l)$.

Let U be a complex Banach space of finite dimension. By a U-polynomial we mean a polynomial function on $\mathbf{C}^l$ with values in U. Consider the space $\mathcal{E}(U)$ of all endomorphisms of U and put $|T| = \sup_{|u| \leq 1} |Tu|$ $(u \in U)$ for $T \in \mathcal{E}(U)$. In this way $\mathcal{E}(U)$ also becomes a Banach space. If $p(\mathbf{t}) = \sum_{\mathbf{k}} u(\mathbf{k})\mathbf{t}^{\mathbf{k}}$ $(u(\mathbf{k}) \in U, \mathbf{k} \in Z_+{}^l)$ is a U-polynomial, we define $\|p\| = \sum_{\mathbf{k}} |u(\mathbf{k})|$.

Let $\mathbf{C}_+{}^l$ denote the set of all points $\mathbf{t} \in \mathbf{C}^l$ such that $\mathrm{Re}\, t_i > 0$ $(1 \leq i \leq l)$. Put $\mathbf{R}_+{}^l = \mathbf{C}_+{}^l \cap \mathbf{R}^l$.

THEOREM 1. *Let Γ_i $(1 \leq i \leq l)$ be holomorphic functions on $\mathbf{C}_+{}^l$ with values in $\mathcal{E}(U)$. Suppose V is a nonempty connected open set in $\mathbf{R}_+{}^l$ and ϕ a function on V with values in U, which satisfies the system of differential equations $\partial\phi/\partial t_i = \Gamma_i(\mathbf{t})\phi$ $(1 \leq i \leq l)$ on V. Then there exists exactly one holomorphic function Φ on $\mathbf{C}_+{}^l$ such that 1) $\Phi = \phi$ on V and 2) $\partial\Phi/\partial t_i = \Gamma_i(\mathbf{t})\Phi$ $(1 \leq i \leq l)$ on $\mathbf{C}_+{}^l$.*

The proof depends on the fact that $\mathbf{C}_+{}^1$ is a simply connected subdomain in $\mathbf{C}$.

Now let $c_i(\mathbf{k})$ $(1 \leq i \leq l, \mathbf{k} \in \mathbf{Z}_+{}^l)$ be elements in $\mathcal{E}(U)$ such that $\sum_i \sum_{\mathbf{k}} |c_i(\mathbf{k})| e^{-s(\mathbf{k})\eta} < \infty$ for every $\eta > 0$. Put

$$C_i(\mathbf{t}) = \sum_{\mathbf{k}} c_i(\mathbf{k}) e^{-\langle \mathbf{k}, \mathbf{t} \rangle} \qquad (\mathbf{t} \in \mathbf{C}_+{}^l).$$

Then obviously C_i is a holomorphic function on $\mathbf{C}_+{}^l$ with values in $\mathcal{E}(U)$. Let $W(\varepsilon, \eta)$ $(0 < \varepsilon < 1, \eta > 0)$ denote the set of all $\mathbf{t} \in \mathbf{C}^l$ such that $\mathrm{Re}\, t_i > \max(\varepsilon |\mathbf{t}|, \eta)$ $(1 \leq i \leq l)$.

THEOREM 2. *Let Φ be a holomorphic function on $\mathbf{C}_+{}^l$ such that $\partial\Phi/\partial t_i = C_i(\mathbf{t})\Phi$ $(1 \leq i \leq l)$. Then we can choose a set Λ of points in $\mathbf{C}^l$ and, for each $\lambda \in \Lambda$, a U-polynomial p_λ such that the following conditions are fulfilled.*

(1) $p_\lambda \not\equiv 0$ for every $\lambda \in \Lambda$.

(2) For any ε, η $(0 < \varepsilon < 1, \eta > 0)$ and $\mu \in \mathbf{C}^l$, the series $\sum_\lambda |p_\lambda(\mathbf{t}) e^{\langle \lambda + \mu, \mathbf{t} \rangle}|$ converges uniformly on $W(\epsilon, \eta)$.

(3) $\Phi(\mathbf{t}) = \sum_\lambda p_\lambda(\mathbf{t}) e^{\langle \lambda, \mathbf{t} \rangle}$ $(\mathbf{t} \in \mathbf{C}_+{}^l)$.

Moreover the set[2] Λ and the polynomials p_λ $(\lambda \in \Lambda)$ are uniquely determined by these conditions.

Let us call an element $\lambda \in \Lambda$ maximal if $\lambda + \mathbf{k}$ does not lie in Λ for any $\mathbf{k} \not\equiv 0$ in $\mathbf{Z}_+{}^l$. Let Λ_0 denote the set of all maximal elements in Λ.

THEOREM 3. *Λ_0 is a finite set and every element in Λ is of the form $\lambda_0 - \mathbf{k}$ where*

$\lambda_0 \in \Lambda_0$ *and* $\mathbf{k} \in \mathbf{Z}_+^l$. *Moreover the degrees of the U-polynomials* p_λ $(\lambda \in \Lambda)$ *remain bounded and we can choose positive numbers* a, b *such that* $\|p_\lambda\| \leq ab^{-s(\lambda)}$ *for every* $\lambda \in \Lambda$.

We call the function $\Phi_0(t) = \sum_{\lambda \in \Lambda_0} p_\lambda(t) e^{<\lambda, t>}$ the principal part of Φ. Notice that $\Phi_0 \not\equiv 0$ unless $\Phi = 0$.

We shall now present some applications of these results to the theory of spherical functions on a connected semisimple Lie group G. Suppose the center of G is finite and K is a maximal compact subgroup of G. Let $\sigma = (\sigma_1, \sigma_2)$ be a (continuous) double representation of K on a finite-dimensional (complex) vector space U and let V be an open set in G such that $KVK = V$. By a spherical function ϕ on V of type σ, we mean a continuous function with values in U such that $\phi(k_1 x k_2) = \sigma_1(k_1)\phi(x)\sigma_2(k_2)$ for $x \in V$ and $k_1, k_2 \in K$. Put[3] $G^+ = KA_\mathfrak{p}{}^+K$. Then G^+ is open in G.

LEMMA 1. *Suppose* $V \subset G^+$ *and* ϕ *is a spherical function on* V *of type* σ *and class* C^∞ *such that* ϕ *is an eigenfunction of every differential operator in*[3] $\mathfrak{Z}$. *Then if* V *is connected and nonempty,* ϕ *can be extended to an analytic function on* G^+ *which is also spherical of type* σ.

This is a consequence of Theorem 1.

Let π be a quasi-simple[4] irreducible representation of G on a Banach space B. Let $\mathfrak{d}$ be an equivalence class of finite-dimensional irreducible representations of K and $E_\mathfrak{d}$ the canonical projection[4] in B corresponding to $\mathfrak{d}$. We assume that $\mathfrak{d}$ is so chosen that $B_\mathfrak{d} = E_\mathfrak{d}B \neq \{0\}$. Let $\pi_\mathfrak{d}(k)$ $(k \in K)$ denote the restriction of $\pi(k)$ on $B_\mathfrak{d}$. Then we have a double representation $\sigma = (\sigma_1, \sigma_2)$ of K on $\mathcal{E}(B_\mathfrak{d})$ given by $\sigma_1(k_1)T\sigma_2(k_2) = \pi_\mathfrak{d}(k_1)T\pi_\mathfrak{d}(k_2)$ $(T \in \mathcal{E}(B_\mathfrak{d}); k_1, k_2 \in K)$. Put $\phi(x) = E_\mathfrak{d}\pi(x)E_\mathfrak{d}$ $(x \in G)$. Then ϕ can be regarded as a spherical function on G of type σ. Let M denote the centralizer of[3] $A_\mathfrak{p}$ in K and $d(\mathfrak{d})$ the degree of any representation in $\mathfrak{d}$.

THEOREM 4. *Dim* $B_\mathfrak{d} \leq d(\mathfrak{d})^2$ *and we can choose a linear function* λ *on*[3] $\mathfrak{h}_p$ *and an element* $S \in \mathcal{E}(B_\mathfrak{d})$ *such that* S *commutes with* $\pi_\mathfrak{d}(m)$ $(m \in M)$ *and*[3]

$$\phi(x) = \int_K \pi_\mathfrak{d}(k_x) S \pi_\mathfrak{d}(k^{-1}) e^{\lambda(H(xk))} dk \qquad (x \in G).$$

Here dk *is the normalized Haar measure on* K.

This is an extension of an earlier result.[5] The proof depends on the notion of the principal part introduced above.

Let τ be a representation of K on a finite-dimensional space U. Then we can define a double representation $\sigma = (\sigma_1, \sigma_2)$ of K on $\mathcal{E}(U)$ as follows: $\sigma_1(k_1)T\sigma_2(k_2) = \tau(k_1)T\tau(k_2)$ $(k_1, k_2 \in K, T \in \mathcal{E}(U))$. Let $\mathcal{E}_0$ denote the set of all elements in $\mathcal{E}(U)$ which commute with $\tau(m)$ for every $m \in M$. Fix $T \in \mathcal{E}_0$ and consider the function[3]

$$\phi_{H'}(x) = \int_K \tau(k_x) T \tau(k^{-1}) \exp\left\{(-1)^{1/2} \langle H', H(xk)\rangle - \rho(H(xk))\right\} dk$$

for $H' \in \mathfrak{h}_\mathfrak{p}$ and $x \in G$. Then for a fixed H', $\phi_{H'}$ is a spherical function of type σ. Theorem 2 enables us to obtain a formula[6] for $\phi_{H'}$ which expresses its asymptotic behaviour very clearly.

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[1] Rec denotes the real part of a complex number c.

[2] Note that Λ is empty in case $\Phi = 0$.

[3] See *Amer. Jour. Math.*, **80**, 241–310 (1958), for the meaning of undefined symbols.

[4] See *Trans. Amer. Math. Soc.*, **75**, 185–243 (1953).

[5] See *Trans. Amer. Math. Soc.*, **76**, 63 (1954).

[6] This is a generalization of Lemma 37 of the paper in reference 3.

SPECTRAL REPRESENTATIONS AND UNBOUNDED CONVOLUTION OPERATORS

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1. Let S be a locally compact space and $M(S)$ the vector space of complex Radon measures on S with compact support. For any compact $K \subset S$ let $M(S, K)$ be the Banach space (endowed with the usual norm) of all measures belonging to $M(S)$ and whose support is contained in K. Let us consider on $M(S)$ the inductive limit topology of the topologies of the spaces $M(S, K)$.

Let $\mathfrak{A}$ be a vector subspace of $M(S)$. Suppose that $\mathfrak{A}$ is endowed with the multiplication $(\nu, \mu) \to \nu\mu$ and that, for this multiplication, $\mathfrak{A}$ is an algebra. A continuous complex-valued function χ, defined on S is a *character* of $\{S, \mathfrak{A}\}$ if $\chi \neq 0$ and if $\int_S \chi d\nu \int_S \chi d\mu = \int_S \chi d\nu\mu$ for any $\nu, \mu \in \mathfrak{A}$. Let us denote by E the set of all characters of $\{S, \mathfrak{A}\}$ endowed with the topology of uniform convergence on compact subsets of S, be $\tilde{E}$ the Stone-Cech compactification of E and by E' the set of real-valued $\chi \in E$. Let $\mathfrak{M}$ be the set of all locally bounded positive functions defined on S and for each $\mathfrak{r} \in \mathfrak{M}$ let $E(\mathfrak{r}) = \{\chi \| \chi(s)| \leq \mathfrak{r}(s) \text{ for } s \in S\}$.

Suppose now that there is a part $\mathfrak{L} \subset \mathfrak{A}$ having the properties: (1.1) if f is a continuous complex-valued function defined on S and if $\int_S f\, d\,\nu = 0$ for every $\nu \in \mathfrak{L}$, then $f = 0$; (1.2) for each $\mu \in \mathfrak{L}$ there is a continuous mapping $s \to \mu_s$ of S into $\mathfrak{A}$ such that $\int_S f d\nu\mu = \int_S d\nu(s) \int_S f d\mu_s$ for every $\nu \in \mathfrak{L}$ and every continuous complex-valued function f defined on S. Then:

Proposition 1. *$E(\mathfrak{r})$ is a locally compact subspace of E for each $\mathfrak{r} \in \mathfrak{M}$.*

2. For each $\mathfrak{r} \in \mathfrak{M}$ let $S_1(\mathfrak{r})$ be the set of all objects $\{X, U_s\}$ where: (2.1) X is a Banach space; (2.2) $s \to U_s$ is a weakly continuous mapping of S into the set of scalar operators (with strongly completely additive spectral measure) on X, such that $\|U_s\| \leq r(s)$ for each $s \in S$; (2.3) the ranges of the spectral measures of the operators U_s, $s \in S$, are contained in the same σ-complete boolean algebra of projections;[1] (2.4) $\int_S U_s d\nu(s) \int_S U_s d\mu(s) = \int_S U_s d\nu\mu(s)$ for every $\nu, \mu \in \mathfrak{A}$.

Let N_2 be the set of all objects $\{X, U_s\}$ where: (2.5) X is a Hilbert space; (2.6) $s \to U_s$ is a mapping of S onto a set of commuting, not necessarily bounded, normal operators on X; (2.7) $s \to (U_s x|y)$ is continuous for $x \in \Delta = \bigcap_{s \in S} D(U_s)$ and $y \in X$; (2.8) $(U_\nu U_\mu x|y) = (U_{\nu\mu} x|y)$ for any[2] $\nu, \mu \in \mathfrak{A}$ and $x, y \in \bigcap_{\nu,\mu \in \mathfrak{A}} D(U_\nu U_\mu)$; (2.9) $\bigcap_{s \in S} D(U_s) = \bigcap_{s \in S_0} D(U_s)$ for some denumerable part $S_0 \subset S$.

THEOREM 1. a) *For every $\{X, U_s\} \in S_1(\mathfrak{r})$ there is a spectral family*[3] $\mathfrak{F} = (m_{x,x'})_{x \in X,\ x' \in X'}$ *on $E(\mathfrak{r})$ and only one such that*

$$\langle U_s x, x' \rangle = \int_{E(\mathfrak{r})} \chi(s) dm_{x,x'}(\chi) \text{ for all } s \in S,\ x \in X,\ x' \in X'.$$

The spectral measure $P_\mathfrak{F}$ corresponding to $\mathfrak{F}$ verifies the equation $P_\mathfrak{F}(E(\mathfrak{r})) = I$ if and only if for each nonzero $x' \in X'$ there is a v such that ${}^t U_v x' \neq 0$. b) For every $\{X, U_s\} \in N_2$ there is one and only one hermitian spectral family[3] $\mathfrak{F} = (m_{x,y})_{x \in X, y \in X}$ *on $\tilde{E}$, concentrated on E, and such that*

$$(U_s x|y) = \int_{\tilde{E}} \chi(s) dm_{x,y}(\chi) \text{ for all } s \in S,\ x \in D(U_s),\ y \in X.$$

The spectral measure $P_{\mathfrak{F}}$ corresponding to $\mathfrak{F}$ verifies the equation $P_{\mathfrak{F}}(E) = I$ if and only if for each nonzero $x \in \Delta$ there is a v such that $U_v x \neq 0$.

If, in the statement a), U_s is supposed to be normal, for each $s \in S$, then $\mathfrak{F}$ is hermitian spectral. If, in the statement b), U_s is supposed to be hermitian, for each $s \in S$, then $\mathfrak{F}$ is concentrated on E'.

3. Let Z be a locally compact space and for each $z \in Z$ let m_z be a Radon measure on $Z \times Z$. For every $f, g \in \mathcal{K}(Z)$ denote by $f * g$ the function[4] $z \to m_z(f \otimes g)$. Suppose that: (3.1) for each compact $A \subset Z$ there is a compact $K_A \subset Z$ such that $f, g \in \mathcal{K}(Z, A)$[5] implies $f * g \in \mathcal{K}(Z, K_A)$; (3.2) the multiplication $(f, g) \to f * g$ defines on the vector space $\mathcal{K}(Z)$ the structure of a commutative algebra; (3.3) there is a Radon measure m on Z, strictly positive on nonvoid open sets, such that $m(|f * g|) \leq m(|f|)m(|g|)$ for every $f, g \in \mathcal{K}(Z)$; (3.4) there is an involutive homeomorphism $z \to z^*$ of Z onto Z such that[4] $m(\check{f}) = m(\tilde{f})$ and $m((g * \tilde{h})k) = m((k * \tilde{h})g)$ for every $f, g, h, k \in \mathcal{K}(Z)$.

The multiplication $(f, g) \to f * g$ extended by continuity to $L^1(Z, m)$, gives $L^1(Z, m)$ the structure of a commutative Banach algebra; the mapping $f \to \tilde{f}$ extended by continuity to $L^1(Z, m)$ is an involution on $L^1(Z, m)$ if and only if m_z is real for each $z \in Z$. For every part $S \subset Z$ let $L^1(S, Z, m)$ be the set of all $f \in L^1(Z, m)$ which are zero almost everywhere (with respect to m) on the complement of a compact (depending on f) contained in S.

Now let S be a closed part of S and suppose that: (3.5) m is strictly positive on the nonvoid parts of S, open in S; (3.6) $f, g \in L^1(S, Z, m)$ implies $f * g \in L^1(S, Z, m)$. Let $\mathfrak{A} = \mathfrak{L} = \{(f.m)_S \mid f \in L^1(S, Z, m)\}$[6] and for $\nu = (f.m)_S$, $\mu = (g.m)_S \in \mathfrak{A}$ ($g, h \in L^1(S, Z, m)$) define $\nu\mu = ((g * \check{h}).m)_S$; then $\mathfrak{A}$ becomes an algebra. The object $\{\mathfrak{A}, \mathfrak{L}\}$ satisfies (1.1) and (1.2) if we suppose that: (3.7) for given $f \in \mathcal{K}(Z)$ and compact $A \subset Z$, the mappings $z \to m_z(f \otimes g)$, with $g \in \mathcal{K}(Z, A)$ and $\|g\|_\infty \leq 1$, are equicontinuous.

4. Consider the object $\{Z, m_z, m, (f, g) \to f * g\}$ introduced above and suppose that the conditions (3.1)–(3.4) are satisfied and that: (4.1) m_z is real for each $z \in Z$; (4.2) there is a $e \in Z$ such that $e^* = e$ and $m_e(f \otimes \bar{g}) = m(f\bar{g})$ for any $f, g \in \mathcal{K}(Z)$.

Let $\mathfrak{A} = \{f.m \mid f \in L^1(Z, Z, m)\}$, define the multiplication on $\mathfrak{A}$ as in section 3 and denote by $\hat{Z}$ the set of all bounded characters of $\{Z, \mathfrak{A}\}$ satisfying the relation $\bar{\chi} = \chi$; then $\hat{Z}$ ($\subset E(1)$) is locally compact for the topology $\sigma(L^\infty(Z, m), L^1(Z, m))$.

For each $f \in \mathcal{K}(Z)$ let T_f be the function defined by $T_f(\chi) = \int_Z \chi(z)f(z)dm(z)$, $\chi \in \hat{Z}$. On $\hat{Z}$ there is a (unique) measure $\hat{m}$ such that $T: f \to T_f$ may be extended[7] to an isomorphism of $L^2(Z, m)$ onto $L^2(\hat{Z}, \hat{m})$.[8] The mapping $T: f \to T_f$ may also be extended[7] as a one-to-one continuous mapping of $L^p(Z, m)$ ($1 \leq p \leq 2$) into $L^{p'}(\hat{Z}, \hat{m})$ ($1/p + 1/p' = 1$) and $\|T_f\|_{p'} \leq \|f\|_p$ for each $f \in L^p(Z, m)$.

Suppose now: (4.3) m_z positive for every $z \in Z$. Let $\mathfrak{M}$ be the set of complex-valued m-measurable functions defined almost everywhere on Z (we identify two functions which are equal almost everywhere). Let $f, g \in \mathfrak{M}$. By definition[9] $f * g$ exists if $f \otimes g$ is m_z-integrable for almost every $z \in Z$ and if $z \to \int_{Z \times Z} |f \otimes g| dm_z$ is locally m-integrable; $f * g(z) = \int_{Z \times Z} f \otimes g dm_z$ almost everywhere on Z. For each $f \in \mathfrak{M}$ let D_f be the set of all $g \in L^1(Z, m) \cap L^2(Z, m)$, such that $f * g$ exists and belongs to $L^2(Z, m)$. Then $U_f: g \to f * g$ is a linear operator with domain D_f. Denote by G_f the closure of the graph of U_f and by $\bar{U}_f$ the closure of U_f, whenever it exists.

THEOREM 2. *Consider the following propositions: 1)* $\bar{D}_f = L^2(Z, m)$ *and* $\bar{U}_f$ *exists; 2)* $\bar{U}_f$ *exists and is normal; 3) there is a complex-valued* $\hat{m}$*-measurable function* T_f' *defined on* $\hat{Z}$ *such that* $(g, h) \in G_f$ *if and only if* $g, h \in L^2(Z, m)$ *and* $T_h = T_g T_f'$*; 4) there is a set* $D \subset D_f$ *of functions with compact support such that the vector space spanned by the set of functions* $g * h$*, where* $g \in D$ *and* $h \in \mathcal{K}(Z)$*, is dense in* $L^2(Z, m)$*. Then 1), 2) and 3) are equivalent and 4) implies 1) = 2) = 3). If 1) = 2) = 3) is valid then* $T\bar{U}_f T^{-1}$ *is the multiplication operator by* T_f' *on* $L^2(\hat{Z}, \hat{m})$*. For* $f \in L^p(Z, m)(1 \leq p \leq 2)$ *1) = 2) = 3) is valid and* $T_f' = T_f$ *locally almost everywhere.*

5. The results in section 2 show that the representation theorems obtained by the first-named author in *J. of Math. and Mech.*, **8,** 95–110 (1959) remain valid under the more general conditions considered here.[10] Concerning the conditions (3.5), (3.6) and the object $\{\mathfrak{A}, \mathfrak{L}\}$, introduced in section 3, see also a paper of the second-named author in *Trans. Am. Math. Soc.*, **92,** 154–167 (1959). Concerning the definition of $\{Z, m_z, m, (f, g) \to f * g\}$ and the conditions (3.1)–(3.4), (3.7) and (4.1)–(4.3) see Berezanski, I. M., and S. G. Krein, *Uspehi Math. Nauk*, **12,** 147–152 (1957), and a review by Godement, R., *Math. Reviews*, **12,** 188–189 (1951). Theorem 2 extends the main results given by Kunze, R. A., in *Ann. of Math.*, **69,** 1–14 (1959).

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[1] See Dunford, N., *Pacific J. Math.*, **4,** 321–354 (1954), and Bade, W. G., *Trans. Am. Math. Soc.*, **80,** 345–360 (1955).

[2] U_δ ($\delta \in M(S)$) is defined by: $(U_\delta x \mid y) = \int_S (U_s x \mid y) d\delta(s)$ for $x \in \Delta$ and $y \in X$.

[3] For the definition see *J. of Math. and Mech.*, **8,** 95–110 (1959). For other definitions and notations see Bourbaki, N., *Intégration* (Paris, 1952–1957), Chap. 1–5.

[4] The functions $\tilde{f}$ and $g \otimes h$ are defined respectively by: $\tilde{f}(z) = \overline{f(z^*)}$ for $z \in Z$ and $g \otimes h((x, y)) = g(x)h(y)$ for $(x, y) \in Z \times Z$.

[5] For each compact $A \subset Z$ we write $\mathcal{K}(Z, A) = \{f \in \mathcal{K}(Z) \mid \text{support } f \subset A\}$.

[6] $f.m$ is defined by: $m_f(g) = \int_Z g f dm$ for $g \in \mathcal{K}(Z)$, $(f.m)_S$ is the measure on S induced by $f.m$.

[7] We use the same notation for the extended mapping.

[8] An inversion formula similar to the one valid in the case of a commutative locally compact group [Loomis, L. H., *An Introduction to Abstract Harmonic Analysis*, (New York, 1953), p. 143] may be obtained if (3.7) is satisfied.

[9] The definition given here for $f * g$ is compatible with the definition given in section 3. If $f, g \in M$ and if one of them is m-negligible then $f \otimes g$ is m_z-negligible for every $z \in Z$.

[10] See also Hille, E., and Phillips, R. S., *Functional Analysis and Semi-groups* (Providence: 1957), Chap. 22; Nussbaum, A. E., *Ann. of Math.*, **69,** 133–141 (1959); and Levitan, B. M., Translation 59, *Am. Math. Soc.* (1951).

LOWER BOUNDS FOR RISK FUNCTIONS IN ESTIMATION*

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1. Introduction.—Let T be an estimator of θ, of a probability density function $p(x, \theta)$ where $x = (x_1, \ldots, x_n)$ is a vector of observations on a (vector or sequence) random variable (*r.v.*)X, and θ is an unknown parameter (a constant). If $R(T, \theta) = E_\theta W(T, \theta)$ is the risk function, where E_θ stands for the mathematical expectation when θ is the true parameter, and $W(T, \theta)$ is the loss in choosing T when θ is the true value, then it is of considerable interest in theoretical statistics to obtain the best lower bounds for $R(T, \theta)$, without actually evaluating it.[1–5] The actual computation of $R(T, \theta)$ is much more difficult since it is related to the solution of the distribution problem of T. If $W(T, \theta)$ is quadratic or $W(T, \theta) = |T - \theta|^p$, $p \geq 1$, several lower bounds have been obtained in the past,[1–4] most of them, using the unbiased estimators, T of θ. In this paper, all these results are unified and extended without assuming unbiasedness. Non-sequential problems only are considered. As usual, the *r.v.*'s are denoted by capitals, and the values assumed by them by the corresponding small letters.

2. Assumptions.—Let $\theta = (\theta_1, \ldots, \theta_k)$ be a point in A, a bounded non-empty subset of a real Euclidean k-space. For any x in $\bar{S}$, also a real Euclidean space and θ in A, there exists a density $p(x, \theta)$ with respect to a fixed σ-finite measure μ defined on the σ-algebra of subsets of $\bar{S}$. It will be assumed that the carrier of $p(x, \theta)$, say $S(\subset \bar{S})$, remains invariant for all θ in A. Further the following regularity conditions are imposed on $p(x, \theta)$.

Condition I: All partial derivatives of $p(x, \theta)$ with respect to θ_i $(i = 1, \ldots, k)$ exist for all x in S and θ in A. (For later analysis, arrange these derivatives in some order. For definiteness, group the first order derivatives first, then the second order etc., and within groups use a lexicographic ordering. Then eliminate all those terms that are linearly dependent on the preceding ones, and divide the resulting sequence by $p(x, \theta)$. Denote the i^{th} term by $D_i(x, \theta)$ or D_i for short.

Condition II: For each i, $|p(x, \theta)D_i(x, \theta)| < M_i(x)$ and $\int_S M_i(x)d\mu < \infty$ all θ in $\bar{A}$ (closure of A).

Condition III: The *r.v.*'s D_i, $i = 1, 2, \ldots$, are square integrable $(p(x, \theta)d\mu)$.

Condition IV: If the column vector $T = (T_1, \ldots, T_k)'$ (prime for transpose) is an estimator of θ', where the T_i have a finite second moment and $E_\theta(T_i) = \theta_i + b_i(\theta) = \alpha_i(\theta)$ with $b_i(\theta)$ as bias, then all the partial derivatives of $\alpha_i(\theta)$ exist with respect to the θ_i.

It is possible to state several other sets of conditions in place of the set I–IV above.

3. Quadratic Loss.—The loss functions $W(T, \theta)$ to be considered here are of the following two types.

$$W_\delta(T, \theta) = [\sum_{i=1}^{k} (T_i - \theta_i)^2]^{\delta/2}, \; 1 < \delta \leq 2, \text{ and } 2 < \delta \leq 4, \tag{1}$$

$$W_a(T, \theta) = \sum_{i,j=1}^{k} (T_i - \theta_i)(T_j - \theta_j)a_i a_j, \tag{2}$$

where a_i are arbitrary real numbers (non-stochastic and known). Note that if T is unbiased, then $R_a(T, \theta) = E_\theta W_a(T, \theta)$, is the reciprocal of the familiar concentration ellipsoid given by Cramér.[3]

Let $f_m^{(j)} = D_{(m-1)k+j}, j = 1, \ldots, k$, and $f_m = (f_m^{(1)}, \ldots, f_m^{(k)})', m = 1, 2 \ldots$. (If the sequence of column vectors $\{f_m\}$ is finite, and the last vector happens to be incomplete, then add zeros to complete it.) Let $\mathfrak{L}^2$ be the space generated by all finite linear combinations of $\{f_m\}$, i.e., f_m, f_n in $\mathfrak{L}^2$ implies $Af_m + Bf_m$ in $\mathfrak{L}^2$ for all $(k \times k)$ constant matrices A and B. If the resulting space is not complete, add the limit points of all such combinations under the norm $\|f_m\|^2 = tr(f_m, f_n) = \sum_{i=1}^{k} E_\theta |f_m^{(i)}|^2$, where $(f, g) = (E_\theta(f^{(i)}g^{(j)}))$.

Using the classical Schmidt procedure the $\{f_m\}$ sequence can be orthogonalized, and denote by $\{\varphi_m\}$, the orthonormal sequence so obtained. Let $A_n = (T, \varphi_n)$. From the above assumptions, A_n can be calculated explicitly. If $\delta = 2$ in (1), one obtains the following

THEOREM 1. *Suppose the density function $p(x, \theta)$ satisfies the conditions I–IV of section 2. Let T' be a (row) vector of estimators and θ the true parameter value in A. If $R(T, \theta) = E_\theta W(T, \theta)$ where $W(T, \theta)$ is given by (1) with $\delta = 2$, or (2), then the corresponding lower bounds are given by*

$$R_2(T, \theta) \geq \sum_{n=1}^{\infty} \operatorname{tr}(A_n A_n'), \tag{3}$$

$$R_a(T, \theta) \geq \sum_{n=1}^{\infty} a'(A_n A_n')a, \tag{4}$$

where $a' = (a_1, \ldots, a_k)$. The lower bounds in (3) and (4) are reached if and only if $(T - \theta')$ lies in $\mathfrak{L}^2$. The minimum risk estimator is essentially unique.

The above result enables one to obtain the following interesting

COROLLARY 1.1 *In order that the lower bounds in (3) or (4)* (and hence those given by others[2-4]) *for $R(T, \theta)$, $\delta = 2$, may be reached, it is necessary that the estimator T of θ' be unbiased.*

COROLLARY 1.2. *If a sufficient statistic, for θ, exists, then, when $T - \theta'$ lies in $\mathfrak{L}^2$, T is a (Borel) function of the sufficient statistic.*

The above statements are proved with the help of certain simple facts of the $\mathfrak{L}^2$ space. In particular, a vector analog of the Bessel-inequality is employed.

It will be noticed that all the classical results[2-4] on these inequalities are contained in (3). If T is unbiased, the results on the concentration ellipsoid[3-5] are subsumed under the bound given in (4).

In the case $1 < \delta < 2$, many difficulties arise to give bounds corresponding to (3) or (4), and restrictive assumptions on $p(x, \theta)$ are needed. If δ is of a certain form, then the restrictions are somewhat reasonable.

THEOREM 2. *Let $\delta = 2q/(2q - 1), q \geq 1$ integer, and μ be the Lebesgue measure. Let $D_i\sqrt{p(X, \theta)}$ and $(T_j - \theta_j)\sqrt{p(X, \theta)}$ be in $L^\delta(S, \mu)$ for each i, j (i.e. $\int_S d_i{}^\delta p(x, \theta)^{\delta/2} d\mu$, and $\int_S (T_j - \theta_j)^\delta p(x, \theta)^{\delta/2} d\mu$ exist), and suppose that $M = \underset{x \in S}{l\,u\,b}\, p(x, \theta)^{(\delta-2)/2} < \infty$, for all θ in A. If the conditions I–IV of section 2 hold, then*

$$R_\delta(T, \theta) = E_\theta[\sum_{i=1}^{k} (T_i - \theta_i)^2]^{\delta/2} \geq M^{-1}[\sum_{n=1}^{\infty} |A_n^q|_E^2]^{\delta/2q}, \tag{5}$$

where $|A|_E^2 = tr(AA')$, A_n *is defined before, and* $A^q = (a_{ij}^q)$ if $A = (a_{ij})$.

Remark: If $q = 1$, then $\delta = 2$, and $M = 1$ so that (5) coincides with (3). Analogous result, if $2 < \delta \leq 4$, is given by the following

COROLLARY 2.1. *Let the risk function be* $\bar{R}_\delta(T, \theta) = E_\theta[\sum_{i=1}^{k} (T_i - \theta_i)^2]^{\delta}$, *with* $1 < \delta \leq 2$, *and the density function* $p(x, \theta)$ *satisfy a Lipschitz condition of order* α $(0 < \alpha \leq 1)$. Let $\delta = 2q/(2q - 1)$, $q \geq 1$ *integer, and* $D_i\sqrt{p(X, \theta)}$ *and* $(T_i - \theta_i)\sqrt{p(X, \theta)}$ *be in* L^δ *with* μ *as Lebesgue measure. If the conditions I–IV of section 2 hold, then*

$$\bar{R}_\delta(T, \theta) \geq (\int sp(x, \theta)^{\delta-1} d\mu)^{-1} (\sum_{n=1}^{\infty} |A_n^q|_E^2)^{r/q}. \tag{6}$$

The theorem and its corollary are proved upon employing the result of Theorem 1, and an extension of a special form of Hausdorff-Young Theorem[7] for the vector variables.

The requirement that μ be a Lebesgue measure is not quite essential, and μ can be, for instance, a counting measure. However, it is necessary that it be translation invariant to obtain the bounds (5) and (6).

If δ is allowed to vary continuously, further restrictions on $p(x, \theta)$ should be imposed. That bound will not be presented here as the result obtained is not very pleasing.

4. Convex Loss: The most interesting type of loss functions can be subsumed under the following type. Let $y = t - \theta$, and $W_\theta(y)$ stand for a function whose argument depends only on $(t - \theta)$, but which may contain θ in some other manner. Let $W_\theta(y)$ satisfy the conditions: (a) $W_\theta(y) \geq 0$, for all θ in A, and y, (b) $W_\theta(0) = 0$, (c) $W_\theta(y) = W_\theta(-y)$, (d) $W_\theta(y)$ is convex in y for each θ in A, and is measurable in both y and θ, and (e) $E_\theta W_\theta(\bar{T}) < \infty$ for all θ in $\bar{A}$, where $\bar{T} = T - \theta$, and T is an estimator of the (single) parameter θ.

It will be noted that, if $W_\theta(y)$ satisfies the conditions (a)–(e), there exists a function $V_\theta(y)$ satisfying (a)–(e) and such that $V_\theta^k(y) = W_\theta(y)$ for some $k(\geq 1)$. Let k_0 be the largest such value of k.

THEOREM 3. *Let T be an estimator of* θ, *in A, the true parameter. Suppose* $p(x, \theta)$ *satisfies conditions I–IV of section 2, for* $i = 1$ (i.e. *there is only* D_1), *and* $D_1^{k'}$ *is integrable* $(p(x, \theta)d\mu)$, *where* $k^{-1} + k'^{-1} = 1$, *and* $1 \leq k \leq k_0$. *If the loss function* $W_\theta(y)$ *satisfies the conditions* (a)–(e) *above, then*

$$R(T, \theta) = E_\theta(W_\theta(\bar{T})) \geq W_\theta\left(\frac{1 + b'(\theta)}{E_\theta(|D_1|)}\right)[E_\theta(|D_1|)/E_\theta^{k'_0-1}(|D_1|^{k'_0})]^{k_0}, \tag{7}$$

where if $k_0 = 1$, $E_\theta^{1/k'_0}(|D_1|^{k'_0})$ *is taken as the ess. sup. of* $|D_1|$, *and* $b(\theta)$ *is, as usual, the bias with* $b'(\theta) = \dfrac{db(\theta)}{d\theta}$.

This theorem is proved using several inequalities on convex functions, and then showing that the second factor in square brackets on the right of (7) is a monotone

increasing function of k. If $W(y) = |y|^2$, (7) reduces to the classical Cramér-Rao inequality.[3, 4] Other specializations lead to certain other results.

5. An Extension: A further extension of the convex loss is the following which includes some results of Barankin.[1] Let $\varphi(t)$ and $\psi(t)$ be two non-negative symmetric convex functions such that $\varphi(0) = \psi(0) = 0$, and

$$|ab| \leq \varphi(a) + \psi(b), \text{ for any real } a \text{ and } b. \tag{8}$$

It is seen that φ and ψ satisfy the conditions (a)–(e) of the preceding section. Define the risk function as

$$R_\varphi(T, \theta) = \|\bar{T}\|_\varphi = \sup_{d_i} \int s|\bar{d}_i| p(x, \theta)d\mu, \text{ with } \int s\psi(d_i)p(x,\theta)d\mu \leq 1. \tag{9}$$

($\|D_i\|_\psi$ is defined similarly.) If $\varphi(u) = |u|^k/k$ and $\psi(u) = |u|^{k'}/k'$ where $k^{-1} + k'^{-1} = 1$, and T is unbiased, then $R_\varphi(T, \theta) = \|\bar{T}\|_\varphi = k'^{1/k'}\|T\|_k = k'^{1/k'}R_k^{1/k}(T, \theta)$. Barankin[1] has obtained lower bounds for $R_k^{1/k}(T, \theta)$. The risk function in (9), therefore, includes his.

Let L^φ and L^ψ be the classes of all *r.v.*'s $\bar{T}$ and D_i and all of their finite linear combinations such that $\|\bar{T}\|_\varphi$ and $\|D_i\|_\psi$ are finite. It is known that these spaces are complete (or are completed by adding the limit points under their respective norms) linear spaces.

THEOREM 4. *Suppose $M(\bar{T})$ is the subspace of L^φ that contains the elements $\bar{T}(= T - \theta)$, where T is an estimator of θ, of $p(x, \theta)$. Then (i) $M(\bar{T})$ is non-empty if and only if there exists a positive constant K (independent of n) such that for every set of r.v.'s $D_{i_1}, \ldots, D_{i_n}$ in L^ψ, where $\varphi(u)$ and $\psi(u)$ satisfy (9) and $\varphi(2u) \leq C\varphi(u)$, where C is a finite positive constant independent of u, and for any set of n real numbers $a_1, \ldots, a_n$, the following inequality obtains:*

$$\left|\sum_{j=1}^{n} a_j \alpha^{ij}(\theta)\right| \leq K\left\|\sum_{j=1}^{n} a_{ij} D_{ij}\right\|_\psi, \tag{10}$$

where $\alpha(\theta) = E_\theta(T)$, and $\alpha^i(\theta) = \dfrac{d^i\alpha(\theta)}{d\theta^i}$, (ii) for every $\bar{T}$ in $M(\bar{T})$, $\|\bar{T}\|_\varphi \geq C_0$, where $C_0 = g\ l\ b\ K$ satisfying (10), and (iii) if there is a $\bar{T}^*$ in $M(\bar{T})$ such that $\|\bar{T}^*\|_\varphi = C_0$, then it is essentially unique.

This theorem is a consequence of some results in functional analysis and the Zaanen representation theorem[6] for linear functionals in the L^φ spaces.

The details of the proofs, illustrations, and some extensions will be published separately.

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ORBIT SPACES OF ABELIAN p-GROUPS*

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1. Let p be a prime and S a cohomology n-sphere[1] over Z_p (the integers mod p). Let π_a be an abelian group of order p^a and type $(p, \ldots, p)$ acting effectively on S to form a transformation group (S, π_a). Let S_f be the π_a-free part of S, that is, the union of orbits of cardinality p^a. For $\pi_b \subset \pi_a$, denote by $F(\pi_b)$ the fixed-point set of π_b. $F(\pi_b)$ is a cohomology sphere over Z_p. Let $n(\pi_b) = \dim_p F(\pi_b)$. Then $n(\pi_b) \leqq n$ and if $p \neq 2$, $n - n(\pi_b)$ is even. Cyclic subgroups π_1 of π_a which are not in π_b act effectively on $F(\pi_b)$. If U is π_b-invariant, denote by $P(U, \mu_b) = P(U, \pi_b, t)$ the Poincaré polynomial of the cohomology of the orbit space U/π_b over Z_p. It is known[1] that for $a = 1$,

$$P(S_f, \pi_1) = P(n(\pi_1) + 1, n) \tag{1.1}$$

where $P(h, k) = t^h + t^{h+1} + \ldots + t^k$. We conjecture that if $a \geq 2$, then for any subgroup π_{a-1} of π_a,

$$(1 - t)P(S_f, \pi_a) = \sum_{\pi_1 \not\subset \pi_{a-1}} P(F_f(\pi_1), \pi_{a-1}) - tP(S_f, \pi_{a-1}) \tag{1.2}$$

where,[2] in $P(A_f, \mu)$, A_f means the μ free part of A. Since π_a is abelian, π_{a-1} acts on $(F\pi_1)$.

We shall sketch a proof for the case $a = 2$. It can be shown that for $a \geq 2$, (1.2) implies the relations

$$\sum_{\pi_{a-1} \subset \pi_a} (n(\pi_{a-1}) - n(\pi_a)) = n - n(\pi_a) \tag{1.3}$$

which have been established by Borel.[3]

From now on take $a = 2$ and denote π_2 by π. The subgroups $\pi_{a-1} = \pi_1$ are cyclic, $p + 1$ in number; denote them by $\alpha = \alpha_1, \alpha_2, \ldots, \alpha_{p+1}$. Let $n_i = n(\alpha_i)$, $n_0 = n(\pi)$. The formula to be proved is

$$(1 - t)P(S_f, \pi) = \sum_{i=2}^{p+1} P(F_f(\alpha_i), \alpha) - tP(S_f, \alpha)$$

which, as a consequence of (1.1) and the fact that the fixed-point set of α_i $(i \geq 2)$ acting on $F(\alpha)$ is precisely $F(\pi)$, can be written

$$(1 - t)P(S_f, \pi) = \sum_{i=2}^{p+1} P(n_0 + 2, n_i + 1) - tP(n_1 + 1, n) \tag{1.4}$$

$$= \sum_{i=1}^{p+1} P(n_0 + 2, n_i + 1) - P(n_0 + 2, n + 1) \tag{1.4$'$}$$

The following explicit formula can be obtained readily from (1.4):

$$P(S_f, \pi) = t^{n_0+2} \sum_{i=2}^{p+1} P(0, q_1 + \ldots + q_{i-1} - 1)P(0, q_i - 1),$$

where $q_i = n_i - n_0$. The relations (1.3) for $a = 2$ come from putting $t = 1$ in (1.4).

2. *An exact sequence.* Let β be a cyclic group of order p and g a generator. In the group ring $Z_p(\beta)$ let $\sigma = 1 + g + \ldots + g$, $\tau = 1 - g$. We have the relations $\sigma\tau = \tau\sigma = \sigma\sigma = 0$. Now let Y be a free $Z_p(\beta)$-module which is a cochain complex and let Y^β denote the set of β-invariant elements. Evidently $Y^\beta = \sigma Y$.

Let ρ be either one of σ, τ and $\bar{\rho}$ the other. From now on, every statement involving ρ, $\bar{\rho}$ will hold equally when ρ, $\bar{\rho}$ are interchanged.

There exists an exact sequence

$$0 \xrightarrow{\rho} \rho Y \to Y \xrightarrow{\bar{\rho}} \bar{\rho} Y \to 0$$

giving rise to the cohomology sequence

$$\ldots \to H_\rho^{k-1} \to H^k \xrightarrow{i^{*k}} H^k \xrightarrow{\rho^{*k}} H_\rho^k \to H_\rho^{k+1} \to \ldots \tag{2.1}$$

where $H^k = H^k(Y)$, $H_{\bar{\rho}}^k = H^k(\bar{\rho}Y)$, ... Let $b_\rho^k = \dim H_\rho^k$, $c_\rho^k = \dim(Ker\ \rho^{*k})$, $e_\rho^k = \dim(Im\ \rho^{*k})$. Then

$$b_\rho^{k+1} = b_{\bar{\rho}}^k + c_{\bar{\rho}}^{k+1} - e_{\bar{\rho}}^k$$

which, putting $B_{\bar{\rho}} = \sum_k b_\rho^k t^k$ etc. can be written

$$tB_\rho = tB_{\bar{\rho}} + C_{\bar{\rho}} - tE_{\bar{\rho}}. \tag{2.2}$$

Note that B_σ is the Poincaré polynomial (or series) for $H(\sigma Y) = H(Y^\beta)$.

3. If $\mathrm{H}^n = Z_p$, *the composition of the maps*

$$\mathrm{H}^n \xrightarrow{\rho^{*n}} \mathrm{H}_\rho^n \xrightarrow{i^{*n}} \mathrm{H}^n \tag{3.1}$$

is zero. For let $u \in H^n$ and let z be a cocyle in u.

Then ρz represents the image of u in H_ρ^n, hence it also represents the image of u under the composition map. Since $\mathrm{H}^n = Z_p$, β acts trivially on H^n, hence ρz cobounds, so ρz represents the zero of H^n.

4. *The cohomology of* S_f/α. Let U_f, U_h denote the π-free and α-free parts of U (see §1). *From now on the index* i *will be restricted to the range* $2, \ldots, p+1$.

Let $F_i = F_h(\alpha_i)$, $F = \cup F_i$. One sees that the F_i are disjoint and so $H(F/\alpha) = \Sigma H(F_i/\alpha)$, direct sum. Moreover,

$$S_f = S_h - F,\ S_f/\alpha = S_h/\alpha - F/\alpha.$$

Using (1.1), the cohomology sequence for the pair $(S_h/\alpha, F/\alpha)$ yields

$$H(S_f/\alpha) = {}^1H + \Sigma\, {}^iH; \qquad {}^1H = i^*H(S_h/\alpha); \qquad {}^iH = \delta^*H(F^i/\alpha) \tag{4.5}$$

where i^* is an isomorphism and δ^*, the connecting homomorphism, raises dimensions by 1 and is injective. The easiest way to verify this is to assume that $n_i \leqq n_1$ which is permissible since the final result (1.4)′ is independent of the order of the α's.

5. There is a natural effective action of $\beta = \pi/\alpha$ on S_f/α and it is readily verified that the fixed-point set of β is F/α. We assert that *each element* w *of* ${}^iH$ *contains a cocycle which* β*-invariant.* It is sufficient to show that $w \in pr^* H((S_f/\alpha)/\beta)$ where pr is the natural mapping $S_f/\alpha \to (S_f/\alpha)/\beta$ and this last follows from the commutative

$$\begin{array}{ccccc} H(F/\alpha) & \xrightarrow{\delta^*} & H(S_h/\alpha - F/\alpha) & = & H(S_f/\alpha) \\ \| & & \uparrow pr^* & & \\ H((F/\alpha)/\beta) = H(F/\alpha) & \to & H((S_h/\alpha)/\beta - F/\alpha) & = & H((S_f/\alpha)/\beta) \end{array}$$

As a consequence of the preceding paragraph and the relation $\rho\sigma = 0$ (see §2) we have that

$$\rho^*({}^iH) = 0, \qquad i = 2, \ldots, p+1, \tag{5.1}$$

where $\rho^* = \Sigma\rho^{*k}$.

6. We use S_f equally to denote the Alexander-Spanier cochains of S_f with compact carriers and values in Z_p. Let $W = \mathbf{C} \otimes S_f$ where $\mathbf{C}$ is an *acyclic* α-free cochain complex over $Z_p(\alpha)$ on which β acts trivially.[4] Let α and β act diagonally on W.

Now let z be a cocycle of $\mathbf{C}^\alpha$. The endomorphism ξ_z of the complex W defined by $\xi_z(\Sigma c_j \otimes s_j) = \Sigma_z \cdot c_j \otimes s_j$ where $z \cdot c_j$ is the cup product, permutes with α and therefore defines an endomorphism of W^α. One verifies that ξ_z permutes or antipermutes with the differential operator (depending on the degree of z), hence induces an endomorphism of $H(W^\alpha)$. Now β acts on W^α and is seen to permute with ξ_z. Hence ρ^* (defined for $Y = W^\alpha$, §2) permutes with ξ_z. *It follows that*

$$\rho^* w = 0 \text{ implies } \rho^*(\xi_z w) = 0, \ (w \in H(W^\alpha)). \tag{6.1}$$

7. *From now on take* Y *in* §2 *to be* W^α. From the known facts about tensor products[5] we have

$$H(W) = H(S_f);\ H(Y) = H(S_f^\alpha) = H(S_f/\alpha);$$
$$H(Y^\beta) = H(S_f^\alpha)^\beta = H(S_f^\pi) = H(S_f/\pi).$$

Since $Y^\beta = \sigma Y$ *we can compute* $H(S_f/\pi)$ *as* $H(\sigma Y)$, *and* $P(S_f, \pi)$ as B_σ(§2).

We first obtain $H(Y)$. A simple spectral sequence argument[6] yields an additive base for $H(Y)$ of the form

$$y^{s+n_1+1} = c^s \otimes u^{n_1+1} \qquad s = 0, \ldots, n - n_1 - 1 \tag{7.1}$$

$$i_y{}^{s+n_0+2} = c^s \otimes {}^iu^{n_0+2} \qquad \begin{array}{l} s = 0, \ldots, n_i - n_0 - 1, \\ i = 2, \ldots, p+1, \end{array} \tag{7.2}$$

where $c^s \in H^s(\mathbf{C}^\alpha)$, $u^{n_1+1} \in H^{n_1+1}(S_f)$, ${}^iu^{n_0+2} \in {}^iH^{n_0+2}(S_f)$. There exists[7] in the algebra $H(\mathbf{C}^\alpha) = H(\alpha)$ an element $v = v(s)$ of degree $n - n_1 - 1 - s$ such that $v \cdot c^s$ is a *nonzero* element of degree $n - n_1 - 1$ (when $p \neq 2$, this depends on the fact that $n - n_1$ is even so that one of the integers s, $n - n_1 - 1 - s$ is even). Let $z(s)$ be a cocycle in $v(s)$. We then have

$$\xi_{z(s)} y^{s+n_1+1} = \lambda y^n \qquad (0 \neq \lambda \in Z_p). \tag{7.3}$$

8. We next show that for $Y = W^\alpha$,

$$H_\rho{}^n = H_{\bar\rho}{}^n = Z_p, \qquad Im\,\rho^{*n} = Im\,\bar\rho^{*n} = Z_p. \tag{8.1}$$

For j sufficiently large, the groups $H_\sigma{}^j = H(Y^\beta) = H(S_f/\pi)$ are trivial since S_f/π is finite dimensional because S_f is. Hence from (2.1), $H_\rho{}^j = H_{\bar\rho}{}^j = 0$ for $j > n$, and ρ^{*n}, $\bar\rho^{*n}$ are surjective. Suppose $\rho^{*n} = 0$. Then in (2.1), i^{*n} is surjective. Hence

the composition map in (3.1) is surjective. The composition map can therefore not be zero since (7.2) shows that $H^n(Y) = Z_p$. Since we thus obtain a contradiction to the statement in §3, we conclude that $\rho^{*n} \neq 0$, $\bar{\rho}^{*n} \neq 0$. It follows that $H_\rho{}^n \neq 0$, $H_{\bar{\rho}}{}^n \neq 0$ and (8.1) now follows from $H^n = Z$, $H_\rho{}^{n+1} = H_{\bar{\rho}}{}^{n+1} = 0$.

9. *Determination of C and E.* Referring to the base (7.1) (7.2) we assert that

$$\rho^* y^{s+n_1+1} \neq 0; \qquad \rho^*({}^i y^{s+n_0+2}) = 0. \tag{9.1}$$

Suppose $\rho^* y^{s+n_1+1} = 0$. Then by (6.1) $\rho^*(\xi_{z(s)} y^{s+n_1+1}) = 0$, i.e., $\rho^* y^n = 0$ which, since y^n is a generator of $H^n(Y)$ contradicts (8.1). This proves the first part of (9.1). As for the second, $\rho^*({}^i y^{s+n_0+2}) = \rho^*(c^s \otimes {}^i u^{n_0+2}) = c^s \otimes \rho^*({}^i u^{n_0+2}) = 0$ by (5.1).

Consequence of (7.1) (7.2) (9.1) (see §2 for notation):

$$c_\rho{}^k = \sum_i {}^i c_\rho{}^k, \; {}^i c_\rho{}^k = 1, \; k = n_0 + 2, \ldots, n_1 + 1$$

$$e_\rho{}^k = 1, \; k = n_1 + 1, \ldots, n \qquad (\rho = \sigma, \tau).$$

All other c's and e's are zero. It follows that

$$C_\rho = C_{\bar{\rho}} = \sum_i P(n_0 + 2, \ldots, n_i + 1)$$

$$E_\rho = E_{\bar{\rho}} = P(n_1 + 1, \ldots, n)$$

Formula (1.4) now follows from (2.2) and the fact that

$$P(S_f, \pi) = B_\sigma \text{ as remarked in §7.}$$

* This work has been supported by the Office of Naval Research.

[1] We follow the definitions in Borel, "Novelle démonstration d'un théorèm de P. A. Smith," *Commentarii Math. Helv.*, 29 (1955). We assume implicitly that the cohomology relations $H(B \bmod A) = H(B - A)$ hold for certain pairs (A, B) constructed from S. It would for example be sufficient to assume that S is metrizable, but certain weaker conditions can be used. Cohomology is based on Alexander-Spanier cochains with compact supports.

[2] We write $F_f(\ )$ for $(F(\ ))_f$. In $P(A_f, \pi)$, A_f is the π-free part of A.

[3] Borel, *Seminar on Topological Transformation Groups*, (Institute for Advanced Study, 1958–59), Chap. 13.

[4] Tensor products are over Z_p.

[5] See for example the reference in footnote 3, also Cartan, H., *Seminaire de Topologie Algebrique de l'E. N. S.*, (Paris: 1951–52).

[6] Let E_r be the spectral sequence for the covering $S_f \to S_f/\alpha$. Then E_∞ is associated with $H(Y^\alpha)$. The cocycles listed form a base for a subgroup of E_2 which consists of permanent cocycles and projects isomorphically onto E_∞.

[7] See for example the reference in footnote 1.

*HEREDITARY DEFECTS IN GALACTOSE METABOLISM IN ESCHERICHIA COLI MUTANTS, I. DETERMINATION OF ENZYME ACTIVITIES**

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Lederberg and his associates have isolated a number of mutants of *Escherichia coli*, strain K-12. These mutants have defects in galactose metabolism and hence are unable to use this sugar as a carbon source for growth or energy metabolism.[1] Moreover, a virus called lambda which is characteristic for the K-12 strain was found to carry genes for synthesis of enzymes involved in galactose metabolism.[1] It was of primary interest to try to find out whether or not galactose metabolism in the K-12 strains (and in transduced K-12 strains) proceeds along an explored anabolic pathway[2–4] which we shall call the Leloir pathway.

The specific enzymatic methods performed as differential microspectrophotometry for the study of the Leloir pathway[4–6] were shown by one of us (K.K.) to be well applicable to sonicates of *E. coli* K-12 strains.[7] A number of galactose-negative K-12 mutants (isolated by E. and J. Lederberg) were classified according to which enzymes of the Leloir pathway showed a defect. Three different mutants were found to be blocked in galactokinase and four different mutants were blocked in galactose-1-phosphate uridyl transferase.[7]

The experiments to be presented here were performed for various purposes.

(1) A description of enzymatic methods with the intention of studying the physiological role of the Leloir pathway in the K-12 strains. The requirements for specificity made it necessary to develop elaborate and somewhat tedious methods. Once it has been proved that the Leloir pathway dominates the picture it should be possible to streamline the methods. These considerations are particularly relevant to mention in connection with the galactokinase test which is relatively complicated if the insistence on the specificity of the methods is maintained.

(2) To characterize the location of the hereditary block in the Leloir pathway; whether they be single or multiple defects.

(3) To describe the correlation between enzyme defects and the synthesis of other enzymes on a constitutive basis. The enzymes studied include mainly those of the Leloir pathway but also β-galactosidase.

(4) A study of physiological and pathological growth (unbalanced growth) and its dependence on specific hereditary enzyme defects. The phenomenon "galactose induced sensitivity" will be the entire topic of the second paper.

Materials.—*O*-nitrophenol galactoside, β-propyl galactoside, and thiomethyl galactoside were gifts from L. Herzenberg of the National Institute of Allergy and Infectious Diseases; α-galactose-1-phosphate (Gal-1-P)[8] as the barium salt or the dipotassium salt was kindly supplied by D. Broida of Sigma Chemical Company. Some of the Gal-1-P preparations were obtained from E. Maxwell of this Institute and other preparations we owe to the generosity of Z. Hassid and E. Putnam of the University of California and to K. Isselbacher of Massachusetts General Hospital. Uridine diphosphoglucose (UDPG) is a product of Sigma Chemical Company. Uridine diphosphogalactose (UPDGal) was prepared by enzy-

matic means from UDPG and Gal-1-P[6] (kindly prepared by E. Maxwell). Another sample of UDPGal was prepared by one of us (HMK) according to the method of Khorana *et al.*[9,10] Glucose-1,6-diphosphate was generously supplied by L. Leloir of the Institutio de Investigaciones Bioquimicas, Buenos Aires, and V. Najjar of the Vanderbilt University. Uridine diphosphoglucose dehydrogenase (UDPG dehydrogenase) was prepared from calf liver acetone powder according to the method described by Strominger *et al.*[11] Uridine diphosphogalactose-4-epimerase (UDPGal-4-epimerase) was purified from calf liver acetone powder according to the method described by E. Maxwell.[6] Phosphoglucomutase was prepared from rabbit muscle according to the method described by Najjar.[12] Galactose-1-phosphate uridyl transferase (Gal-1-P uridyl transferase) was prepared from calf liver acetone powder.[13] Glucose-6-phosphate dehydrogenase was purified from dried yeast by Kornberg's method.[14]

Bacterial cultures: The bacterial strains used were *E. coli* K-12 generously supplied by E. and J. Lederberg. Most of these mutants were unable to grow on galactose as a carbon source (gal−). The Lederbergs described strains like 3100 (gal+). 3092 (gal−), 3096 (gal−), 3099 (gal−) as isogenic strains differing only in the locus which is affected by a point mutation.[15,16]

Strains 3099, 3264, and 3265 were spontaneous mutants (Lederberg[17]). The other galactose negative K-12 strains were obtained by ultraviolet irradiation.[17] Some of the strains are lysogenic with respect to lambda (and transducing lambda). The lysogeny is subject for a special study by Wiesmeyer and Yarmolinsky (unpublished work). Strain 3104 was obtained through D. Kaiser from E. Lederberg. Strain ML 32,400 was obtained through B. L. Horecker from J. Monod, Institut Pasteur, Paris.[18] Strain C7M stems from Fukasawa and Nikaido, Keio University, Medical School, Tokyo, who have described the strain recently.[19,20]

Methods.—Culture conditions: The cultures were usually obtained as stab culture in tryptone dextrose agar and transferred to slants on tryptone dextrose yeast extract agar or bacto-nutrient agar. They were kept at 0–2°C and frequently transferred. In some cases, for instance, strain 3104 and C7M, slants were prepared from single colonies (derived from single cells). From the slants they were subcultured in a small volume (10 to 30 ml) of liquid medium. In most cases the liquid medium used was ammonium sulphate mineral medium (Medium 56 of Monod *et al.*[21] or Medium A of Hartman[22]). For strain C7M, Bactotryptone broth (Difco) was used. The carbon source used was glycerol in amounts between 0.5 to 1.5 per cent, usually 1 per cent (1×10^{-1} *M*). Glucose was occasionally used as a carbon source in the first subculture in order to speed up growth. However, because of the tendency of glucose to suppress the formation of adaptive enzymes[23] it was usually left out even in the first subculture unless otherwise specified. Galactose was used in rather low concentrations (1×10^{-2} *M* to 1×10^{-4} *M*) and the impurities of glucose (4–5%) which it usually contains were considered negligible. However, in some cases it was found preferable to use purified galactose. This was done by recrystallizing galactose from 70 per cent ethanol solution several times.[24] The cultures which were cultivated for the assay of enzymes were grown in volumes from 200 ml to 300 ml. Cultures of 200 ml or 400 ml medium were shaken at 37°C on a rotatory shaker with favorable aeration.

The cultures were usually harvested when growth had reached 2 to 3 gm. wet weight cells per liter medium. The generation time in a glycerol medium was usually between 60 and 100 minutes. Streaks of such cultures were taken on Eosin Methylene blue agar plates.[25] to ascertain whether the cells were still gal + or gal−. In the former case black colonies develop.[15]

Harvesting and disintegration of bacteria: Cultures of volumes ranging between 200 ml and 2 liters were spun at 0°C in polyethylene containers at 5,900 × g in a Lourdes centrifuge and washed with chilled water. For larger volumes a refrigerated Sharples centrifuge was used. For amounts ranging between 0.5 to 1.5 per cent wet weight of cells the Nossal bacterial cell disintegrator[26] was used. The glass beads (Minnesota Mining and Manufacturing Company Superbrite Glass Beads Type 150) added in amounts of 4 to 6 gm were cooled to −10°C. Phosphate buffer, 0.05 *M*, pH 7.0 was used in amounts of 4 to 6 ml Shaking in the disintegrator placed in an −10° room was stopped every 30 seconds to allow cooling. Usually two 30-second periods sufficed to break the cells. The mixture was subsequently spun for 5 minutes at 11,000 × *g* in the high speed attachment of the International Refrigerated Centrifuge. The pale yellow, slightly turbid supernatant fluid was stored at −20°C.

Determination of growth: Sterile aliquots were drawn from the cultures. Turbidity measurements were performed in a Beckman cuvette (1 cm depth) at 650 mμ. A reading of 0.3 corresponds to 1 × 10^9 cells per ml[27] which again corresponds to 1 mg wet weight of cells per ml.

Protein determination: Protein concentration of cell free extracts was determined by the phenol reagent method.[28]

Detection of sugar in complex polysaccharides: Detection of sugars in complex polysaccharides was performed according to the method of Westphal and Lüderritz.[29]

Quantitative determination of enzyme activity in broken cell preparations. (*1*) *Enzymes of the Leloir Pathway.*

(a) Galactokinase.

Principle of method: The enzyme catalyzes the reaction[1]: ATP + Gal → ADP + *α-Gal-1-P*. The italicized product is the constituent formed in a preincubation mixture which is being quantitatively determined. Fluoride is added in order to protect ATP and Gal-1-P from being dephosphorylated by phosphatase. Addition of magnesium ions serves to insure that the reaction which depends on these ions is not slowed down by a depletion of magnesium due to the presence of fluoride. The Gal-1-P formed is determined in a protein free filtrate by an analytical incubation mixture; the basis for the method rests on its ability to interact with UDPG in the presence of the specific enzyme, Gal-1-P uridyl transferase (used here as indicator enzyme)[10,16]. The sequence of enzymatic reactions used for the analysis of Gal-1-P is as follows:

$$\text{Gal-1-P} + \text{UDPG} \rightleftharpoons \text{G-1-P} + \text{UDP-Gal}$$

$$\text{G-1-P} \rightleftharpoons \text{G-6-P (catalyzed by phosphoglucomutase)}$$

$$\text{G-6-P} + \text{TPN} \rightarrow \text{6-phosphogluconate} + \textit{TPNH} \text{ (catalyzed by G-6-P dehydrogenase)}$$

The absolute molar amount of TPNH formed is used as a direct measure of the galactokinase activity in the preincubation mixture. This is provided that the substrates used in the analytical reactions are in considerable excess of the Gal-1-P stemming from the preincubation mixture.

The determination of the amount of Gal-1-P accumulated in a preincubation setup is based on a preincubation mixture of the following composition: Galactose (recrystallized), 25 μl of a stock solution containing 200 μmoles/ml, i.e., 5 μmoles galactose per ml incubation mixture; ATP (0.1 *M*), 20 μl corresponding to 2 μmoles; Tris buffer (1 *M*, pH 7.5), 100 μl; sodium fluoride (0.5 *M*), 50 μl; $MgCl_2$ (0.1 *M*), 50 μl; H_2O 655 μl; bacterial extract (galactokinase to be determined) 100 μl. Incubation was 0, 10, 20, and 30 minutes. The temperature was 37°C. The reaction was stopped by placing the incubation tube in a boiling water bath for 90 seconds. After spinning, part of the protein-free clear supernatant fluid was analyzed enzymatically for Gal-1-P. Usually a 100 μl filtrate was analyzed.

The *analysis mixture* was composed as follows: UDPG, 20 μl or 0.2 μmoles; G-6-P dehydrogenase, 10 μl of a solution containing 10 mg per ml; TPN, 20 μl of a solution containing 20 mg/ml; cysteine (33 mg/ml, pH 8.5), 100 μl; phosphoglucomutase,[30] 20 μl; $MgCl_2$ (1 *M*), 10 μl; glucose-1,6-diphosphate (0.5 μmole/ml), 5 μl; glycine (1 *M*, pH 8.7), 100 μl; H_20, 610 μl; fractionated Gal-1-P uridyl transferase from liver[13] or from *E. coli* K12 mutant 3092 (galactokinase-less mutant[31]). The amount of filtrate used for the assay was 100 μl. In order to calibrate the system, Gal-1-P in amounts of 0.05 to 0.075 μmoles was added. For each 0.1 μmoles of Gal-1-P per ml a density increase of 0.620 at 340 mμ took place. Usually the G-6-P dehydrogenase was added first in order to determine small amounts of G-1-P or G-6-P present in the filtrate. Subsequently the Gal-1-P uridyl transferase was added and the total density increase at 340 mμ minus the value obtained with a 0 time control of preincubation mixture[32] was a measure of the Gal-1-P which accumulated in the preincubation period and hence of the activity of the galactokinase.

The method is highly specific but not highly sensitive. In its present design it can detect activities of 0.1 to 0.2 μmoles per hour per mg protein. The sensitivity can be increased by analyzing a larger fraction of the preincubation mixture. The latter can also be incubated for longer periods of time (1 and 2 hours). Fluoride has not been found to inhibit galactokinase significantly. Simpler and more sensitive methods for galactokinase can be devised. However, at the initiation of the present project it was essential to demonstrate that the hereditary blocks affected the steps of the Leloir pathway, hence the specific enzymatic methods were deemed most essential.

A new sensitive specific method for Gal-1-P determination has most recently been worked out.[33] In this method human hemolysate (which is free of epimerase) is used as transferase source. The incubation mixture is heat-inactivated and analyzed for consumption of UDPG by means of UDPG dehydrogenase.[11] This method is being modified with the purpose of assaying galactokinase in bacterial extracts.

(b) Gal-1-P uridyl transferase.

Principle of method: The enzyme catalyzes the reaction[4]: Gal-1-P + UDPG $\rightleftharpoons$ *G-1-P* + UDPGal.

The *rate of G-1-P formation* is in this case analyzed by adding the two indicator enzymes and coenzymes in excess, phosphoglucomutase (plus glucose-1,6-diphos-

phate) and G-6-P dehydrogenase plus TPN. The initial *rate of formation of TPNH* is used as a direct measure for the Gal-1-P uridyl transferase activity of the disrupted bacterial cell. Controls having either Gal-1-P or UDPG omitted from the incubation are always used. Reoxidation rate in the extracts is much slower than that of TPN reduction. Yet, for determination of low transferase activities it is necessary specifically to determine the rate of reoxidation of TPNH.

The enzyme activity was determined directly in the Beckman spectrophotometer (i.e., "generation incubation" and "analysis incubation" were combined) by the rate at which TPN was reduced. All constituents, substrates, indicator coenzymes as well as indicator enzymes were added in considerable excess over the transferase to be determined. An incubation mixture for the assay of Gal-1-P uridyl transferase had the following composition: cysteine solution (33 mg per ml pH 8.5), 30 μl; magnesium chloride (0.1 *M*), 10 μl; glycine (1 *M*, pH 8.7), 60 μl; phosphoglucomutase together with glucose-1,6-diphosphate; TPN (20 mg per ml), 10 μl; G-6-P dehydrogenase; UDPG (10 μmole per ml), 20 μl; water, 360 μl. To this mixture is added the K-12 extract in amounts of 50 to 100 μg protein and as the last component Gal-1-P (10 μmole per ml), 30 μl. The total volume is 620 μl. In order to ensure that the indicator enzymes are in excess it may be necessary to add more G-6-P dehydrogenase or phosphoglucomutase or glucose-1,6-diphosphate and ascertain that the rate of release of G-1-P from UDPG and Gal-1-P is not increased. Control mixtures with UDPG without Gal-1-P or Gal-1-P without UDPG should only give very slow release of G-1-P. The release of G-1-P is measured by the rate of increase in optical density change at 340 mμ, using 400 mμ as isobestic point.

Another complication which might obscure comparison on a quantitative basis is the fact that 6-phosphogluconate dehydrogenase present in the lysate (or in some preparations of indicator enzyme glucose-6-phosphate dehydrogenase) will alter the scale. That is, the activity of transferase will be overestimated because each mole of UDPG exchanged will give a deflection at 340 mμ which is double as high. It is therefore necessary always to calibrate the enzyme mixture with a known amount of glucose-1-phosphate or glucose-6-phosphate. A direct assay with 6-phosphogluconate is to be recommended too. In most cases the activity of phosphogluconate dehydrogenase is low. Hence the calibration with glucose-1-phosphate is most important in lysates in which transferase activity is moderate or low. These difficulties are less likely to be encountered if one is dealing with extracts having highly active transferase.

If several factors contribute to complicate the quantitative analysis it might be helpful to perform the assay by a two step procedure. The reaction mixture would in such a case consist of *E. coli* extract, phosphoglucomutase and glucose-1,6-diphosphate, cysteine, $MgCl_2$, Gal-1-P, and UDPG. After a brief incubation (for instance, 5 and 10 minutes) the reaction is stopped and the filtrate is analyzed for glucose-6-phosphate by means of TPN and glucose-6-phosphate dehydrogenase (which should be free of transferase).

The sensitivity of the direct transferase assay is of the order of magnitude of 0.01 μmole per hour per mg protein.

(c) UDPGal-4-epimerase.

Principle of method: The enzyme catalyzes the reaction[1,6]: UDPGal $\rightleftharpoons$ *UDPG*. UDPG formed is determined in a protein-free filtrate by means of a DPN dependent

specific dehydrogenase (UDPG dehydrogenase) which catalyzes the reaction[11]: UDPG + 2 DPN → Uridine Diphosphoglucoronic Acid + 2 DPNH. The latter is performed in an analytical incubation. The *absolute molar amount* of DPNH formed divided with 2 provides a direct measure for UDPGal-4-epimerase activity in the disrupted cells. Since the disrupted K-12 cells do not contain any enzymes which hydrolyze or oxidize UDPG (or UDPGal) it is possible by this method to determine quantitatively even very low activities of UDPGal-4-epimerase.

(a) The two step procedure was performed as follows: The reaction mixture contained glycine (1 *M*, pH 8.7), 30 μl; *E. coli* extract, 20 μl; UDPGal (2.2 μmole/ml), 50 μl; and H_2O, 150 μl. The mixture is incubated for 5 and 10 minutes at room temperature (25°C). At termination the mixture is heat inactivated by placing the tubes (with a marble on top) in a boiling water bath for one and a half minutes. It is then cooled on ice, spun down, and 200 μl of the clear supernatant is used for the analytical mixture. The latter contained glycine (1 *M*, pH 8.7), 70 μl; DPN (25 μmoles per ml), 20 μl; 200 μl supernatant of the preincubated sample; 50 μl water and finally 15 or 20 μl of UDPG dehydrogenase. The control contains the same mixture except that the supernatant stems from a reaction mixture to which UDPGal was added after boiling.

The sensitivity of the epimerase test is of the order of magnitude of 0.005 μmole per hour per mg protein.

(b) The one step direct procedure can be used occasionally for crude extracts but preferentially for fractionated UDPGal-4-epimerase preparations free of hydrogen donors and acceptors. This procedure is executed as follows: The reaction mixture contained glycine (1 *M*, pH 8.7), 50 μl; DPN (25 μmoles per ml), 20 μl; UDPGal (2.2 μmoles per ml.), 30 μl; water, 475 μl; UDPG dehydrogenase 25 μl and *E. coli* extract, 5 or 10 μl. Upon the addition of extract the rate of increase of optical density at 340 mμ is recorded (the isobestic region at 400 to 410 mμ is used for optical density at not too long time intervals). It is important that UDPG dehydrogenase is in excess. This can be ascertained by lowering the amount of *E. coli* extract and seeing if the rate decreases proportionally. The control tube contains the same ingredients with the exception of UDPGal. Reoxidation of DPNH interferes and a direct assay using DPNH as substrate should be performed. Each millimicromole UDPGal converted to UDPG corresponds to an optical density change of 0.020 at 340 mμ.

The sensitivity of the direct epimerase test is as high as that of the indirect.

(2) *β-Galactosidase:*

This enzyme was determined according to the method of Cohn and Monod,[34] using as substrate sodium orthonitrophenol galactoside.

The *rate of increase* of optical density at 420 mμ was followed directly in the Beckman cuvette at 25°C. While the reaction was still proceeding at a linear rate an excess of sodium carbonate (one-fifth volume of 2 *M* sodium carbonate) was added, bringing the pH close to 10. Under these conditions, the enzymatic reaction stops immediately. The optical density at 420 mμ increases by a factor of 1.5 to 2.

The determination of the cellular β-galactosidase was performed in small samples of toluenized bacteria.[35,36] Assays for the enzyme was also performed in the medium. In the assay for β-galactosidase, using toluenized cells or medium, the incubation

temperature was 37°C. The absorption was read at 420 mμ in a Beckman spectrophotometer after the addition of equal volumes of 1 *M* sodium carbonate.

The colorimetric method is identical with that described above. Readings at an isobestic wavelength, for instance, 550 mμ, at the start and at the end of the reaction, so as to correct for dispersion differences due to varying amounts of cells, are to be recommended.

Molar absorbancy index of o-nitrophenol (Eastman Organic Chemical Department) in 0.2 *M* sodium carbonate at 420 mμ (maximum) was determined to be 3,500.

Results and Discussion.—Energy and biosynthetic metabolism of galactose: It is well known that galactose can be metabolized at least in catabolic metabolism in bacteria by another pathway than that of Leloir, namely by direct oxidation, dehydration, and cleavage.[37] Table 1 illustrates that galactose in K-12 *E. coli* is metab-

TABLE 1

PHENOTYPIC DESCRIPTION

Genotype	Medium	Galactokinase μM/mg protein/hr. non-induc.	Galactokinase μM/mg protein/hr. Gal-induc.	Gal-1-P Uridyltransf. μM/mg protein/hr. non-induc.	Gal-1-P Uridyltransf. μM/mg protein/hr. Gal-induc.	4-epimerase μM/mg protein/hr. non-induc.	4-epimerase μM/mg protein/hr. Gal-induc.
W 3100*	A	<0.05	40.0	0.6	5.5	5.0	40.0
W 3100	Br†			0.4	5.8		
W 3092	A	<0.05	<0.05	5.2	4.3	4.0	42.0
W 3078	56	<0.05	<0.05	2.4	2.5		
W 3142	56	<0.05	<0.05	1.4	4.0		+
W 3096	A	0.10	15.0	<0.01	<0.01	2.0	23.0
W 3096′	A	0.20	2.1	<0.1	<0.06		6.4
W 3104	A		+	<0.01	0.1 app.		+
W 3099	A		0.05 app.		0.05 app.	<0.01	<0.01
W 3264	A		0.10		0.35	0.60	0.90
W 3265	A		0.10		0.30	0.50	0.80
ML 32,400	A	<0.1	<0.1		<0.1		+
C7M	Br		+	1.8	9.0	<0.01	<0.01

* The letter W is usually added to the numbers of the K-12 strains. In the text it is left out for the sake of abbreviation.

† Br refers to tryptone broth.

olized practically solely through the Leloir pathway. As appears from the table there are genetic blocks in galactokinase, Gal-1-P uridyl transferase, and in 4-epimerase. A single block in each of these steps is sufficient to render the cells "galactose negative" i.e., unable to use galactose as a carbon source for growth or for acid formation. Some of the strains, namely those with single blocks in transferase are not only galactose negatives but also galactose sensitives presumably due to the accumulation of Gal-1-P and related products (see succeeding article[38]). It is mentioned here because this phenomenon likewise underlines the dominant role of the Leloir pathway. Comments on the doubly defective mutants 3096′ (isolated by selection from 3096 rendered static by galactose) and ML 32,400 will be found in the succeeding article. The effect of "lambda Gal" on enzyme synthesis is under study.

The importance of this pathway for *anabolic* metabolism (synthesis of cell material) is illustrated by the following facts. Mutant 3099 has no detectable amount of 4-epimerase. Consequently one should expect that if this mutant is grown on media which contain no galactose, such as an ammonia mineral medium with gly-

cerol as the sole carbon source, the cell should be devoid of galactose. A preliminary attempt to investigate this point was undertaken. Complex polysaccharides were isolated, hydrolyzed and analyzed. No detectable amount of galactose was found; if present, it must have been in amounts less than 2 per cent of that of glucose. In another epimerase-less strain, C7M, Fukasawa and Nikaido[20] likewise found glucose with no detectable amount of galactose. In contrast to this 3096 which has a block in transferase and a relatively high constitutive 4-epimerase (see Table 1) contains galactose in the ratio of 1:3 as compared with glucose;[39] this corresponds closely to that of the equilibrium of 4-epimerase (Leloir[1]). More work is underway with respect to a characterization of sugars from a number of K-12 mutants.

Inducibility of β-galactosidase and the enzymes of Leloir pathway: The absence of 4-epimerase, as well as of cellular galactose in the 3099 mutant, is contrasted by the ability of administered galactose to induce the formation of β-galactosidase. There is scarcely any induction of galactokinase and transferase. What we want to stress here is that a suspected "galactose-free" cell is nonconstitutive but inducible with respect to β-galactosidase and that galactose can serve as inducer. This poses questions as to the nature of the postulated β-galactosidase repressor,[40] if a specific repressor plays a role here.

The epimerase-less C7M strain behaves vastly differently, almost in the reverse way, from the 3099 epimerase-less strain with respect to inducibility by galactose. In the C7M strain galactose induces the synthesis of galactokinase[20] and of transferase (Table 1; see also succeeding article[38] on galactose induced bacteriolysis) but scarcely, if at all, β-galactosidase. The latter is, however, induced by thiomethylgalactoside (TMG). These results are summarized in Table 2. It

TABLE 2

INDUCIBILITY OF GAL-1-P URIDYL TRANSFERASE, 4-EPIMERASE, AND β-GALACTOSIDASE

Strain	Phenotypic defects (single or triple)	Inducer used	Gal-1-P uridyl transferase Ratio of activities*, induced to non-induced	4-Epimerase Ratio of activities*, induced to non-induced	β-Galactosidase Activity†	β-Galactosidase Ratio of activities induced to non-induced
W 3100	No defect	...	...	...	100	...
		Gal	9	8	1,000	10
W 3092	Single	...	...	...	3	...
		Gal	*I*‡	10	220	73
W 3099	Triple	...	...	...	2.5	...
		Gal	..	...	550	220
W 3264	Triple	...	...	...	3	...
		Gal	..	1.5	500	167
		...	...	...	100‖	...
W 3265	Triple	Gal	..	1.6§	3,000‖	30
		β-PG	..	(1)§	50,000‖	500
C7M	Single	...	...	...	30‖	...
		Gal	5	...	45‖	1.5§
		TMG	..	...	1,400‖	47

* Calculated from the values in Table 1.
† mμmoles ONP liberated per min. per mg protein.
‡ Fully constitutive.
§ Only traces of enzyme activity. The ratio is an approximate value.
‖ Determination on toluenized cells. Activities converted from mμmoles per mg dry weight to protein by assuming that soluble protein constitutes about 50% of the dry weight.

should be added that the poor induction of β-galactosidase by galactose could be due merely to the fact that it is delayed as compared to that of the enzymes of the Le-

loir pathway. The induction of the latter brings about lysis which would prevent any delayed induction of β-galactosidase from manifesting itself. It should also be noted from Tables 1 and 2 that the constitutive level of transferase in C7M is high. Moreover, the induced levels of transferase might have increased further if not interrupted by lysis. The value presented was obtained from an aliquot taken 1 hour after the addition of galactose, shortly before lysis ensued.

Single step mutants with triply defective phenotypes: According to E. Lederberg,[17] the single step spontaneous mutants 3264, 3265, and 3099 are readily reversible and render gal-positive recombinants when intercrossed in a number of combinations (recombinations with single galactokinase-less, transferase-less mutants). Whether 3099 really is a single step can still be debated. If it can be induced to synthesize any epimerase at all the levels are much lower than those of 3264 and 3265. Moreover the results of intercrosses are at the present time less conclusive for this strain since no single epimerase-less K-12 mutant has been identified so far.

The main argument is now the following: How can a genotype representing a single step mutation like 3264 and 3265 manifest itself by a triply defective phenotype? The induced levels of the 3 enzymes of the Leloir pathway are greatly below (only 2 to 5%) those of wild type or single mutants. It should be noted that the constitutive epimerase levels of the same two mutants are definitely lower than those of the other K-12 strains.

The easiest way out would be to assume the existence of a single permease block for galactose. Such a block would have to be a severe one because addition of high amounts of galactose (5% galactose in the glycerol ammonia medium) still does not induce the galactose enzymes nor does galactose in high concentration as the sole carbon source permit even slow growth. The latter observation supports the idea of a permease block. Moreover, the enzyme levels although low should be high enough to permit slow growth with galactose as a sole carbon source. The fact that growth stops completely after the exhaustion of added traces of glucose and of internal carbon reserves speaks in favor of a permease block. The situation would therefore seem fairly clear if it were not for the reason that galactose even in small concentrations elicits a considerable induced synthesis of β-galactosidase in the same strains (e.g., 3264, 3265, and also in 3099).

If we, therefore, were to assume that strains 3264 and 3265 were single mutants, permease-less for galactose, then we would arrive to the paradoxical situation that these cells are permeable for galactose as inducer for β-galactosidase but not for galactose as inducer for the enzymes of the Leloir pathway. One is almost forced into the assumption that the locus for induction of β-galactosidase is in a part of the cell which is accessible to galactose. In contrast the locus which can elicit induction of enzymes of the Leloir pathway is fixed in a less accessible spot for penetration of galactose, at least in the cells with the triple defects.

Degree of constitutivity of the enzymes of the Leloir pathway: As appears from Table 1, the constitutive levels of epimerase are in general high, about 10 per cent of the fully induced levels, whereas those of galactokinase are low, in most cases 1 per cent or less of that of fully induced levels. Constitutivity of transferase varies in an interesting fashion. In the 3100, wild type (gal +) the constitutive levels amount to between 7 and 10 per cent of those of the induced. However, in two of the galactokinase-less mutants the constitutive transferase levels are 100 per

cent or even a little higher than those of the "induced." The constitutivity of epimerase is not affected. The constitutivity of transferase in the galactokinase-less mutants cannot be accounted for by any obvious changes in galactose metabolism (cf. Table 1). True pleiotropism should therefore be considered.

Summary.—A number of mutants of *Escherichia coli* K-12 strains which are defective in galactose metabolism have been identified as having single or multiple defects in the Leloir pathway. Enzyme assays for each step of this pathway have been described. Among the singly defective mutants found can be cited the galactokinase-less and galactose-1-phosphate uridyl transferase-less. A singly defective mutant of uridine diphosphogalactose-4-epimerase of an *E. coli* strain different from K-12 (so called M strain) has also been described. The triply defective mutants (all K-12 strains) are genotypically single mutants (Lederberg).

An attempt to describe the triple defects as a result of a single defect of galactsoe permease is complicated by the fact that galactose although unable to promote induction of any of the enzymes of the Leloir pathway is able to elicit induction of β-galactosidase.

A hereditary block of galactokinase in two mutants of K-12 was found to be correlated with a synthesis of constitutive galactose-1-phosphate uridyl transferse. It seems, however, not possible to derive any correlation between the full constitutivity of galactose-1-phosphate uridyl transferase and the rate of formation of galactose-1-phosphate. Pleiotropism should be considered.

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† Predoctoral Fellow of National Institutes of Health.

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[31] Unpublished methods by K. Kurahashi and A. Sugimura.

[32] It is necessary to set up a zero time control for galactokinase activity and the value for Gal-1-P formed was corrected for the zero time control. This is especially important if liver transferase was utilized as indicator enzyme, since it is contaminated by small amounts of galactokinase.[13]

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*HEREDITARY DEFECTS IN GALACTOSE METABOLISM IN ESCHERICHIA COLI MUTANTS, II. GALACTOSE-INDUCED SENSITIVITY**

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The present study of hereditary galactose sensitivity in bacteria was prompted by the existence of such a pathological state in humans, so-called congenital galactosemia (more correctly, hereditary galactosemia). As is well known, the latter state manifests itself by disease symptoms if the subject receives galactose; later permanent tissue lesions ensue. It was shown [1,2] that the basis for abnormal sensitivity toward galactose is due to a defect in a single enzyme, galactose-1-phosphate

uridyl transferase.[3] The activities of galactokinase and 4-epimerase are within normal range.[4] The biochemical basis for the cellular lesions remains obscure although the accumulation of galactose-1-phosphate[5] is probably an essential feature. The molecular basis for the enzyme defect is totally unexplored.

Hereditary galactose sensitivity exists also in certain mutants of *Escherichia coli*. These strains are unable to metabolize galactose as a sole carbon source, i.e., they are "galactose negatives."

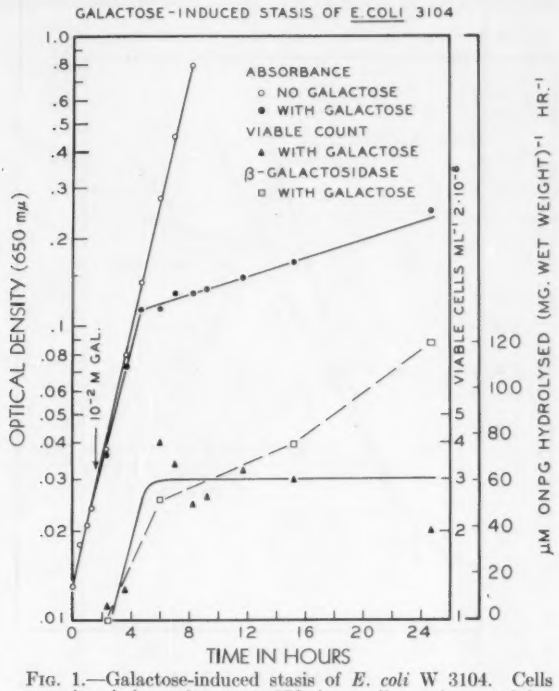

FIG. 1.—Galactose-induced stasis of *E. coli* W 3104. Cells grown in shake culture at 37° in medium A containing 1 per cent glycerol as carbon source. Galactose at a final concentration of 10^{-2} *M* added at the arrow. Viable cells calculated from median values of triplicate plate counts on Difco nutrient agar. β-galactosidase determined on washed toluenized cells by hydrolysis of the chromogenic substate *o*-nitrophenyl-β-D-galactoside.

Among the galactose negatives there are two types of galactose sensitivity: bacteriostasis and bacteriolysis. The sensitivity is undoubtedly dependent on the induced biosynthesis of galactose permease and galactokinase, galactose being the inducer.

Methods and Techniques.—Culture conditions and enzyme assays are described in the preceding article.[6]

Galactose-Induced Bacteriostasis.—A number of galactose-negative K-12 mutants have been shown to lack the enzyme galactose-1-phosphate uridyl transferase[7] and to be inhibited in their growth by the presence of galactose.[8] This inhibition is

very pronounced in a simple minimal medium, such as a mineral ammonia medium, using glycerol as a carbon source, upon the further addition of galactose. In strain 3096[9] grown in glycerol ammonia mineral medium (see previous article[6]), concentrations of galactose as low as 10^{-4} molar suffice to give a strong inhibition of growth.[10]

We want to report some microbiological observations of a galactose-sensitive K-12

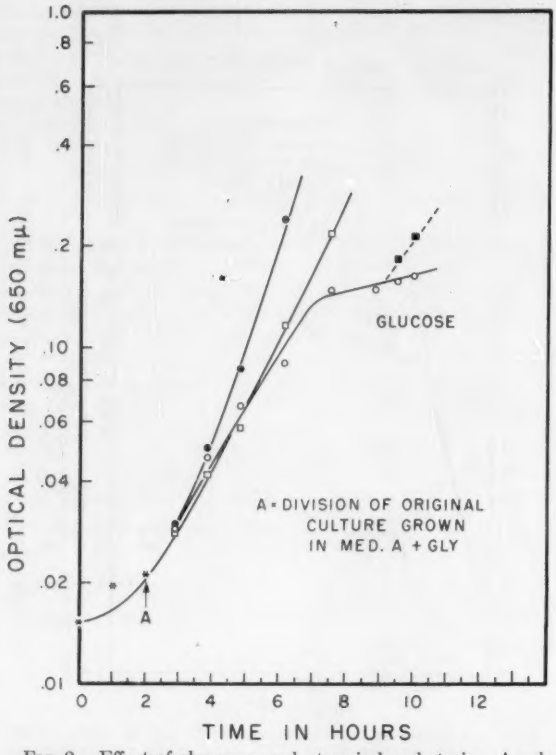

FIG. 2.—Effect of glucose on galactose induced stasis. A culture of W 3104 in medium A with glycerol as a carbon source was started (shake culture, 37°C). At point A the culture was divided into 3 parts. The first culture (●—●) received glucose (10^{-2} *M*); the second culture (□—□) was left unaltered; the third culture (○—○) received galactose (10^{-2} *M*). After 4 hr additional growth the third culture showed stasis. After 2 to 3 hr this culture was divided between two flasks. One flask was left unaltered; to the other flask (■—■) was added glucose (10^{-2} *M*).

strain, 3104. Addition of galactose to this strain brings about an abrupt growth inhibition after about two cell divisions (see Fig. 1).

The inhibition of growth is brought about specifically by galactose; related compounds such as glucose, mannose, and galactosamine or thio-galactosides (which are not hydrolyzed by β-galactosidase) are not inhibitory. The phenomenon is characteristic of the group of mutants blocked at, but not prior to, the transferase. The production of induced galactokinase is probably a prerequisite for growth in-

hibition by galactose in as much as single galactokinase-less as well as the doubly defective galactokinase-less and transferase-less strains do not show any pronounced growth inhibition after addition of galactose.[11]

The galactose inhibition of the transferase-less 3104 was studied in more detail. Viable counts taken at various times during galactose inhibition of growth (Fig. 1) reveal that galactose exerts a bacteriostatic rather than bacteriocidal action.

The apparent stasis might alternatively be explained by assuming that the population remains at all times heterogeneous with respect to its sensitivity to galactose,

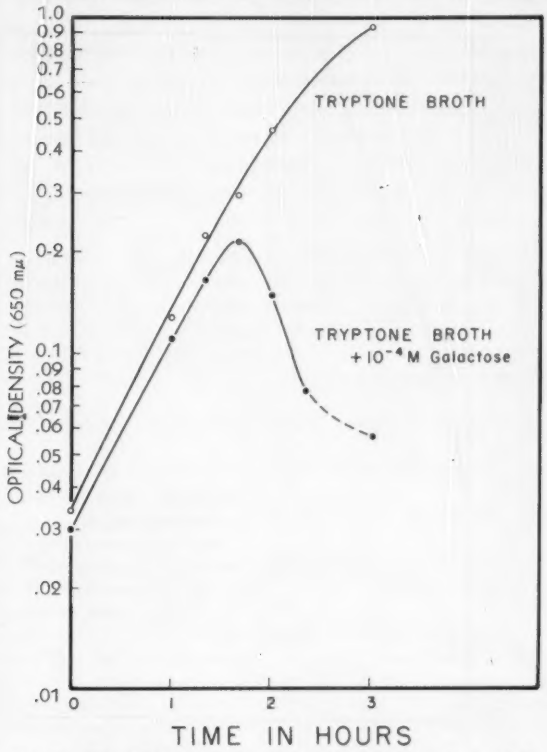

FIG. 3.—The effect of addition of galactose to a culture of *E. coli* strain C7M, growing in tryptone broth (without glucose). The galactose used was D (+) galactose, C. P., Pfanstiehl.

approximately equal proportions being unaffected and being killed, respectively. The following evidence speaks strongly against this hypothesis. The growth-death hypothesis predicts that after very few hours of "stasis" the majority of cells present should become non-viable. Since the absorbance of a culture in "stasis" does not increase rapidly, the non-viable cells must largely fail to contribute to the total turbidity, presumably by virtue of lysing. Lysed cells should release their content of soluble enzymes into the medium. Among these enzymes β-galactosidase, well induced at the onset of "stasis," should be readily detectable. In the experiment of

Figure 1, even after 20 hr of "stasis" the β-galactosidase activity present in the medium amounted to less than one-hundredth the amount found in the cells. Addition of β-galactosidase to the culture showed that the conditions of incubation did not lead to appreciable enzyme inactivation. The foregoing considerations justify the use of the term "galactose-induced bacteriostasis." It is interesting to note that the cellular levels of β-galactosidase increase during the period of stasis (Fig. 1). This is a regular phenomenon in these mutants.

As can be seen from Figure 2 the addition of glucose permits an almost immediate resumption of growth. Within half an hour the generation time goes down from 20

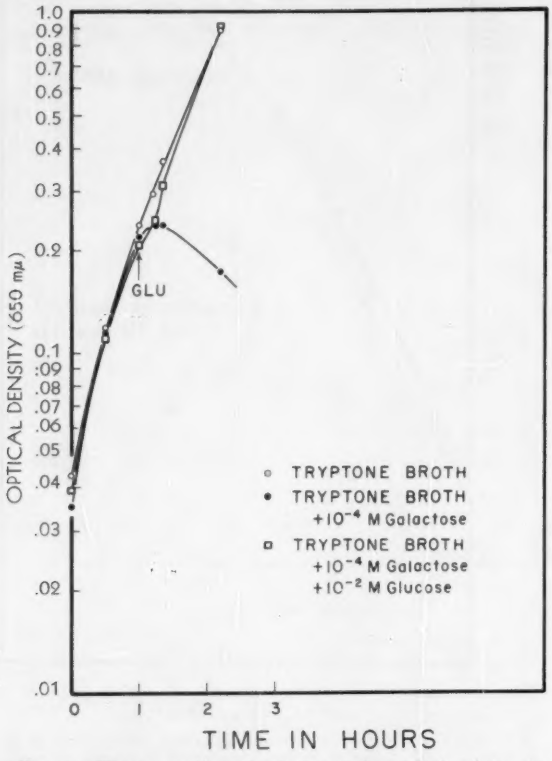

FIG. 4.—The prevention of galactose-induced lysis of *E. coli*, C7M, by glucose.

or 30 hr to about 2 hr. The growth remains logarithmic but fails to attain the rate expected on glucose plus glycerol.

Galactose-Induced Bacteriolysis.—Fukasawa and Nikaido have recently found[12, 13] that galactose causes lysis in a number of galactose-negative mutants of *E. coli* and *Salmonella enteriditis* derived from highly mutable strains (so-called "M" strains). If the cells are in hypertonic medium, spheroplast or protoplast formation ensues.

We have studied some properties of one of these *E. coli* M strains which is known as "C7M."[12–14] In this strain galactose in concentrations as low as 10^{-4} M

causes stril ing lysis even in tryptone broth (see Fig. 3, and ref. 12). As stated in previous communications,[6,13] the "lytic" galactose-negative strains have active galactokinase as well as galactose-1-phosphate uridyl transferase but no trace of 4-epimerase (cells collected and sonicated 1 hr after the addition of galactose but before lysis).

The presence of glucose in addition to galactose in the medium protects the cells against the effects of galactose.[12] It is possible to obtain protection by glucose if it is added 1 hour after the addition of galactose (Fig. 4). At that time, some of the enzymes of galactose-metabolism are induced; the degree of induction is not known.

Accumulation of galactose-1-phosphate takes place during induced stasis as well as prior to and at the onset of lysis.[13] Even in early stasis, levels of 2 to 5.5 μmoles of galactose-1-phosphate per gram cells (wet weight) have been encountered (Spyrides, Jordan, and Kalckar unpublished observations).

Variations in the levels of galactose-1-phosphate, uridine nucleotides and adenyl pyrophosphate compounds during normal. unbalanced, and restored growth are under current study.

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† Postdoctoral Fellow of the National Science Foundation.

‡ Predoctoral Fellow of National Institutes of Health.

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[9] It should be noted that the K-12 strains used are usually preceeded by a "W" *e.g.* W3104.

[10] Kalckar, H. M., and H. de Robichon Szulmajster, *Bull. de Soc. Chim. Biol.*, (in press) (1959).

[11] One can obtain a spontaneous galactose-negative mutant 3096′ as a galactose insensitive strain from the "galactose static" 3096 population. Strain 3096′ is a mutant because it remains insensitive even after passage through galactose-free (non-selective) media. This selected strain was found to be lacking in galactokinase as well as in transferase (cf. [6]).

In addition to these examples can be cited the following additional multiply defective strains which show no sign of galactose sensitivity under the conditions described above: (i) A galactose negative *E. coli* strain ML 32,400 defective in galactokinase and in transferase but having high epimerase; (ii) three triply defective galactose negatives, i.e., having abnormally low galactokinase, transferase and 4-epimerase.

[12] Fukasawa, T., and H. Nikaido, *Nature*, **183**, 1131 (1959).

[13] *Ibid.*, **184**, 1168 (1959).

[14] Our thanks are due to Drs. Fukasawa and Nikaido for making the C7M strain available to us.

*THE RELATIONSHIP OF SODIUM, POTASSIUM, AND DEOXYCORTICOSTERONE IN NEUROSPORA CRASSA**

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Communicated by David M. Bonner, October 12, 1959

The effect of the steroid hormone 11-deoxycorticosterone (DOC) in promoting sodium retention and potassium excretion in mammals is well established, but the mechanism of DOC action in this respect is as yet obscure. Although the kidney appears to be the prominent target for DOC action, this steroid has been shown to influence salt balance, usually as an inhibition of potassium uptake, in other cell types in wide variety of organisms ranging from mammals through amphibia and molluscs to yeasts.[1–3] Similarly, an examination of a large group of steroids for their effect on the growth of *Neurospora crassa* showed that DOC markedly and specifically inhibited the growth of this organism, and also interfered with the uptake of sugars, amino acids, and rubidium.[4] The process of rubidium uptake by *N. crassa* has been intensively examined,[5] and in this organism rubidium uptake appears to be a diphasic phenomenon consisting of a nonspecific but energy-requiring *entry* event, and a specific *binding* event. DOC was found to inhibit the *binding* of rubidium, but had no influence on the *entry* of rubidium.

Since the classical hormonal role of DOC in mammals devolves upon its enhancement of sodium retention, as well as potassium excretion, it was of interest to examine the effect of DOC on sodium transport in *N. crassa*. Thus, the uptake of sodium by germinated conidia of *N. crassa* has been examined with respect to its relationship to potassium and DOC, and the results of these studies indicate that sodium uptake by *N. crassa* involves (a) an *entry* event which, like that of rubidium, is energy dependent, and (b) an *exclusion* system whose effectiveness appears to be related to the saturation or state of the rubidium-potassium *binding* system. The previously demonstrated effect of DOC on the *binding* system appears to be correlated with a more facile and increased penetration of sodium into the *Neurospora* cell, which would seem somewhat analogous to the influence of DOC on the reabsorption of sodium by the kidney.

Materials and Methods.—The system used in these experiments has been previously described,[4] and consisted of a suspension of washed germinated conidia in 20 mM NaH_2PO_4 in 50 mM NaCl, adjusted to pH 5.8 with NaOH. Where indicated, other sodium concentrations were achieved by altering the amount of NaCl; the cells were washed with the same buffered saline solution. Incubations were carried out in a water bath at 30° with agitation. The uptake of sodium was estimated by adding tracer amounts of $Na^{22}Cl$ to the suspension and determining the radioactivity in cell extracts. At various intervals 5.0 ml aliquots of suspension were filtered, with care taken to interrupt filtration when most of the medium had been removed, but before any evaporation of extracellular medium or drying of the cells could occur. The wet cells were weighed immediately and after drying at 100° overnight, the difference representing the total water in the sample of cells. The dried cells were digested with 0.5 ml of 1.0 *N* HCl for 1.0 hr at 125°; then 0.5 ml of 1.0 *N* NH_4OH was added and the digest made up to 3.0 ml with water, mixed

and centrifuged. Aliquots of the supernatant, as well as of the original medium were assayed for radioactivity in a flow-gas counter. Each sample was planchetted in duplicate and each planchette was counted in duplicate or triplicate for a total of at least 8,000 counts.

Since the cells were not washed when harvested it was necessary to make suitable corrections for extracellular sodium in order to assess the sodium in the cells. If the relationship of cell water to dry weight is known then the dry weight can be used to calculate the amount of intracellular water, and this value subtracted from the total water value yields the value for extracellular water. The amount of radioactivity in the medium equivalent to the extracellular water is then subtracted from the total radioactivity in the cell extract to give the value for radioactivity in the cell water. In each experiment one or two separate samples were taken from each flask to determine the cell water-dry weight relationship, using phosphate and inulin to estimate extracellular water.[6] The results for sodium uptake are expressed as the ratio of the Na^{22} concentration in cell water to that in the medium, $[Na^{22}]_i/[Na^{22}]_o$, but these ratios are indicative of the actual uptake of Na^{22}.

The total sodium (mainly Na^{23}) present inside and outside the cells was determined by flame photometry, using lithium as an internal standard. Total sodium was determined in the same samples used for Na^{22} analyses, and the data were treated in the fashion described above.[7] The efflux or uptake of potassium was estimated from the increase or decrease of potassium in the medium and/or in the cells during incubation. Potassium was also determined by flame photometry. Deoxycorticosterone (DOC) was added, were indicated, as a slurry in a small volume of buffer.

Experimental.—With the preparations of germinated conidia used in these experiments the equilibration of sodium between intra- and extracellular phases is achieved in about 2 to 3 hr. As shown in Figure 1, the intracellular concentration of sodium appears to be proportional to the extracellular concentration of sodium, with an average ratio between the two of about 0.82, indicating a slight exclusion of sodium. However, this ratio varies somewhat in different experiments, falling between 0.75 and 1.25; there also appears to be a tendency for these ratios to decrease with an increase in the concentration of external sodium, as can be deduced from Table 1.

TABLE 1

EFFECT OF EXTRACELLULAR SODIUM CONCENTRATION ON THE INTRACELLULAR LEVELS OF SODIUM AND POTASSIUM

Experiment	Incubation Time (hr)	Extracellular		Intracellular	
		Sodium (mM)	Potassium (mM)	Sodium (mM)	Potassium (mM)
I	4.0	22.5	0.15	28	159
		78	0.18	64	161
		208	1.35	175	130
II	3.0	36.5	0.06	33	181
		70.3	0.15	56	179
		141.5	0.51	103	174

* The values for intracellular sodium represent the products of extracellular sodium concentrations (determined by flame photometry) multiplied by the ratios of $[Na^{22}]i/[Na^{22}]o$.

In any case, these cells neither markedly concentrate nor exclude sodium when incubated in buffer containing only various levels of sodium. It can also be seen in Table 1 that large changes in intracellular sodium generally have little effect

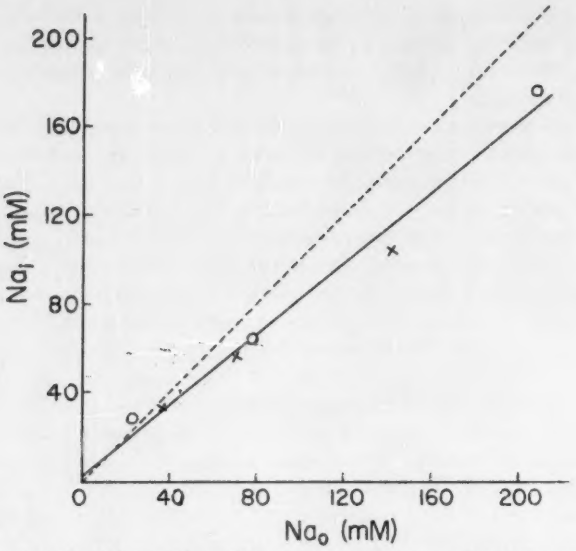

FIG. 1.—Relationship of intra- to extracellular concentrations of sodium at equilibrium. The circles and crosses represent the data from experiments I and II, respectively in Table 1; the solid line has been derived from these points by the method of least squares. The broken line is that expected for equality of intra- and extracellular sodium concentrations.

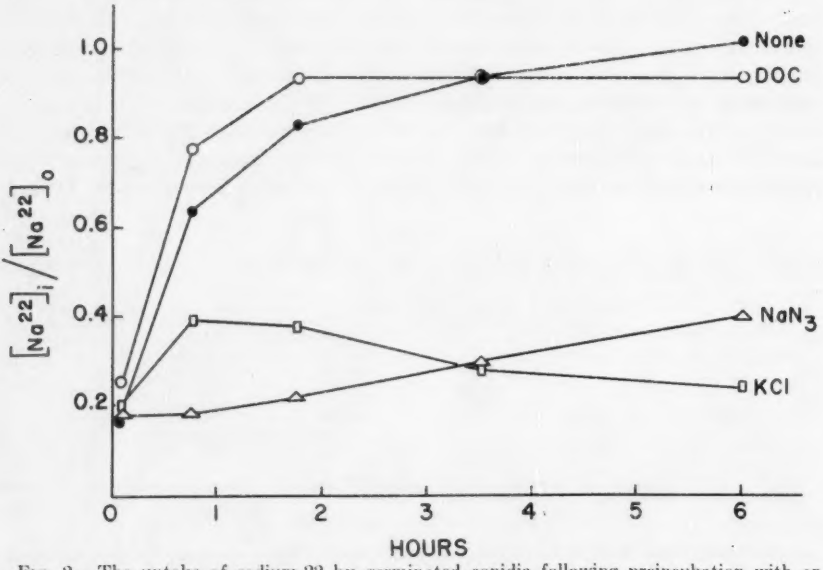

FIG. 2.—The uptake of sodium-22 by germinated conidia following preincubation with or without DOC (0.2 mg/ml), NaN_3 (0.5 mM), or KCl (5 mM). The preincubation times are given with Table 2.

on the concentration of intracellular potassium. Only at the highest concentration of sodium is there obtained a sizable diminution of intracellular potassium, but this decrease in potassium is much smaller than the accompanying increase in sodium. These results resemble certain observations with muscle[8, 9] and bacteria[10, 11] where potassium accumulation appeared to be relatively independent of the intracellular sodium concentration. These results with potassium also complement those obtained in *Neurospora* on the stability of intracellular rubidium.[5]

Sodium uptake has been studied with respect to the effect of various factors which have been shown to have a differential effect on rubidium uptake by *Neurospora crassa*.[5] Thus, azide or dinitrophenol markedly inhibited the *entry* of rubidium into these cells, whereas in the presence of DOC rubidium *entry* was not inhibited, but the attainment of an intracellular concentration of rubidium exceeding that outside the cell (*binding*) was prevented. Potassium did not interfere with the *entry* of rubidium but acted as a competitive inhibitor in the subsequent *binding* of rubidium. These factors have been examined for their effects on the uptake of Na^{22} as well as the intracellular content of total sodium (Na^{23}) and potassium, and the results are presented in Figure 2 and Table 2.

TABLE 2

EFFECT OF AZIDE, DOC, AND KCl ON THE INTRACELLULAR CONCENTRATIONS OF POTASSIUM AND SODIUM

Additions*	Analyses†	Intracellular Concentration (mM) 0 hr	0.75 hr	1.75 hr	3.5 hr.	6.0 hr.
None	K	172	172	172	172	172
"	Na	107	103	107	108	112
NaN_3	K	163	161	158	159	156
"	Na	105	110	106	105	109
DOC	K	133	127	126	122	120
"	Na	135	128	124	127	124
KCl	K	201	215	227	239	254
"	Na	81	73	72	57	55

* The cells were preincubated for 1.5 hr; 1.5 hr with DOC (0.2 mg/ml) or K (5 mM), 0.25 hr with NaN_3 (0.5 mM).

† The values for K and Na were determined by flame photometry in the same samples used for Na^{22} determinations for Figure 2.

With no additions to the system, some variations in the rates of entry of Na^{22} have been observed, with the half-equilibrium times ranging from about 35 to 60 min; the rate of entry of Na^{22} appears to be similar to that observed with rubidium in the presence of DOC.[5] It may also be noted from Table 2 that the content of sodium and potassium in cells incubated in buffered saline alone remains fairly constant. This level of potassium is very close to that observed when the cells were harvested from the germination medium, which has a very low content of sodium ($\sim$1 mM). Since sodium is taken up during the washing of germinated conidia and during the preincubation period, the constancy of the level of intracellular potassium would again indicate that there is not a reciprocal relationship between sodium entry and potassium efflux.

In the presence of azide (0.5 mM) the rate of entry of Na^{22} is markedly reduced, and after 6 hr of incubation the intracellular concentration of Na^{22} is only 0.4 that of the medium. This result is similar to those previously observed with rubidium under similar conditions.[5] The decreased entry rate of Na^{22} does not reflect an expulsion of sodium from the cell since the total sodium concentration within

the cell (Table 2) was unaffected by the presence of azide. Consequently, it would appear that azide might inhibit the efflux of sodium (or rubidium or potassium) as well as its influx. This possibility has been examined and Figure 2*a* shows that the efflux of Na^{22} occurs at a rate similar to its entry, and that this efflux process is also inhibited by azide. The small decrease in intracellular potassium observed in the presence of azide (Table 2) might represent an adverse effect of azide on an energy-dependent potassium *binding* system; however, since efflux as well as entry is energy dependent only a slow potassium efflux would be observed.

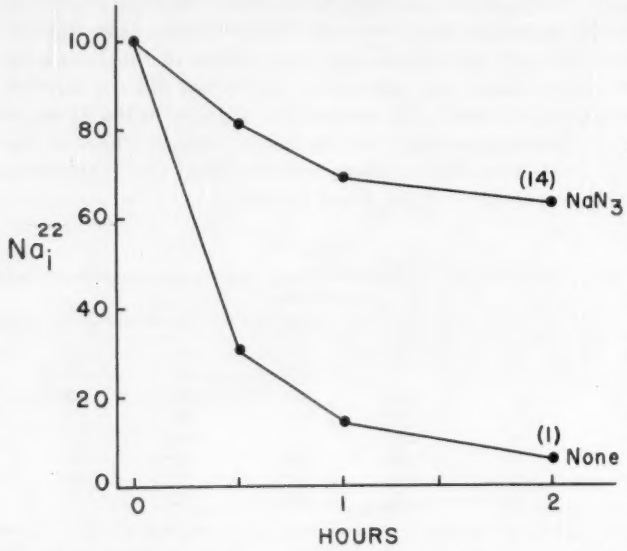

FIG. 2*a*.—The inhibition of efflux of sodium-22 by azide. Germinated conidia were equilibrated with Na^{22}, and then aliquots were filtered and the cells were placed in a similar buffer lacking Na^{22}, with or without NaN_3 (1 mM). Samples were taken at the indicated times, filtered, and the filtrate assayed for Na^{22}; heated samples of suspension were similarly assayed for Na^{22}. The differences between the values for heated and unheated samples were assumed to represent intracellular Na^{22}. The ordinate represents the relative concentrations of intracellular Na^{22}, the zero time value designated as 100. The number in parentheses are the values for $[Na^{22}]_i/[Na^{22}]_0$ at 2 hrs.

The presence of DOC in the system usually results in a somewhat increased rate of entry of Na^{22}, but the equilibrium value obtained is the same as in the absence of DOC. Table 2 shows that only slightly higher levels of Na^{23} are achieved in the presence of DOC than in its absence; this increase becomes maximal during the 1.5 hr preincubation period and remains constant during the subsequent 6 hr of incubation. However, the potassium content of cells exposed to DOC is decreased, indicating that DOC adversely affects the system concerned with the maintenance of high levels of intracellular potassium. This coincidence of an increased rate of sodium entry with a decreased level of intracellular potassium suggests that the rate of entry of sodium is dependent on the degree of saturation or state of the potassium

binding system. These results also suggest the correlative possibility that with increased levels of intracellular potassium the rate of sodium entry would be decreased, and raise the further possibility of sodium exclusion by an intact, saturated potassium binding system.

The likelihood of this possibility is evidenced by the effect of adding potassium to the medium on the entry of Na^{22} and the levels of total intracellular sodium. Figure 2 shows that after preincubation with potassium, the rate of entry of Na^{22} is initially less than that of the control, and during subsequent incubation in the presence of potassium the level of intracellular Na^{22} actually decreases. Similarly (Table 2), the total intracellular sodium has decreased during preincubation, as judged from the zero time values, and continues to fall during subsequent incubation. During this time, the decrease in sodium content is accompanied by increasing levels of intracellular potassium. Thus, sodium exclusion can be clearly demonstrated under conditions where potassium enters the cells and a high intracellular potassium content is achieved.

It is unlikely that the exclusion of sodium can be accounted for by an inhibition of sodium entry by potassium, since it has been demonstrated elsewhere[5] that the entry of so physiologically similar an ion as rubidium is not affected by potassium. On the other hand, the association of sodium exclusion with high levels of intracellular potassium presents the possibility that with increased saturation of a potassium-rubidium *binding* system with potassium less of the cell water is available for sodium. It has been shown previously that with rubidium uptake the *binding* but not the *entry* phase of this process is inhibited by DOC,[5] and, in these studies, that DOC does not inhibit sodium *entry* but decreases the content of intracellular potassium. If the inhibition of sodium uptake and its exclusion results from the *binding* of potassium then DOC should reverse the inhibitory effect of potassium on sodium uptake. This possibility has been examined and the results for Na^{22} are given in Figure 3 and for Na^{23} in Table 3. It can be seen that DOC completely reverses

TABLE 3

EFFECT OF DOC ON THE RELATIONSHIP OF THE INTRACELLULAR CONCENTRATION OF POTASSIUM AND SODIUM

		Intracellular Concentration (mM)			
Additions*	Analyses†	0.5 hr	1.5 hr	2.5 hr	5.0 hr
None	K		174		176
"	Na	96	101	102	104
KCl	K		251		283
"	Na	75	71	67	62
DOC	K		105		101
"	Na	121	117	117	113
KCl + DOC	K		168		170
" "	Na	105	102	107	101

* The cells were preincubated for 2.5 hr; 2.5 hr with DOC (0.2 mg/ml), 1.5 hr with KCl (5 mM).
† The values for K and Na were determined by flame photometry in the same samples used for Na^{22} determinations for Figure 3.

the inhibitory and excluding effect of potassium on sodium uptake. In view of the above considerations these results indicate that the state or degree of saturation by potassium of an intracellular rubidium-potassium *binding* system determines the extent of sodium *exclusion* from the *Neurospora* cell.

Discussion.—The entry of sodium into *Neurospora* appears to occur at a rate similar to that previously observed for rubidium and, like rubidium, the entry of

sodium is markedly inhibited by azide. When its *binding* is prevented by DOC the entry of rubidium is not inhibited by potassium, and under similar circumstances potassium does not inhibit the entry of sodium. In these respects the entry of sodium and rubidium (or potassium) appears to be mediated by a common system which exhibits relatively little specificity for these cations. The *entry* system appears to be the same system involved in the efflux of sodium in so far as both transfers occur at similar rates and both are dependent on metabolic energy for their operation. In this respect it is also evident that in *Neurospora* the *entry* system is not involved in the accumulation or exclusion of potassium (or rubidium) or sodium, respectively, against their concentration gradients. It does not appear likely that the results reported here for sodium and elsewhere[5] for rubidium can be interpreted

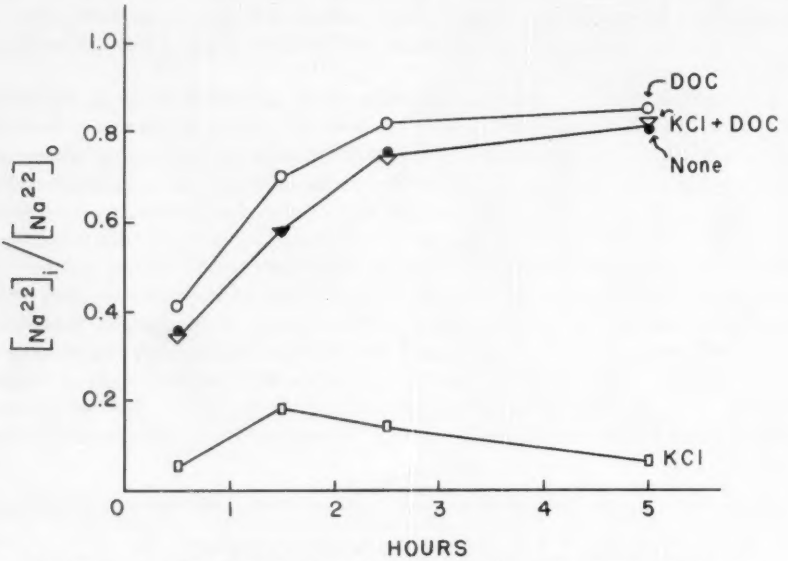

FIG. 3.—The effect of DOC (0.2 mg/ml) on the inhibition of uptake of sodium-22 by potassium (5 mM). The preincubation times are given with Table 3.

on the basis of an hypothesis involving carrier systems in the cell membrane. Such hypotheses necessarily impose a condition of high specificity of membrane carriers, whereas the observations with *Neurospora* indicate a lack of specificity in the *entry* event. The mechanism of *entry* is, as yet, obscure but, as previously discussed,[5] it might be envisaged as involving mechanical actions of the cell membrane[12-14] which are dependent upon a supply of metabolic energy for their functioning. It might be further speculated that the cell surface has a rather passive role, freely permitting the passage of many substances, but that *entry* involves the penetration of contractile lattices or spongelike structures making up a large portion of this cytoplasm.

Despite a relatively high intracellular concentration of potassium, an uptake of sodium can be achieved resulting in an intracellular concentration approximately equal to that outside the *Neurospora* cell. This uptake of sodium is not necessarily

accompanied by an equivalent efflux of potassium, and, indeed, such uptake can be observed under conditions where no significant efflux of potassium occurs. These observations are not in accord with a "sodium pump" hypothesis, since such an hypothesis would appear to require (a) an exclusion of sodium to account for the high ratios of the intra- to extracellular concentrations of potassium observed in these cells, (b) a sizeable efflux of potassium to account for the intracellular levels of sodium which are obtained, and (c) an efflux of potassium and an influx of sodium resulting from azide inhibition of such "pumps." None of these conditions are realized in the case of *Neurospora*, and indeed the observations are quite to the contrary; for example, azide *inhibits both* the entry and efflux of sodium, and causes little change in the intracellular concentrations of potassium.

The exclusion and expulsion of sodium against an apparent concentration gradient can be achieved with *Neurospora* by the addition of potassium to the cell suspensions, which results in a further accumulation of potassium in these cells. As pointed out earlier it is unlikely that the exclusion of sodium results from a competition with potassium in traversing the cell surface, nor, for the reasons stated above, would the aforementioned membrane systems be likely to be involved. The sodium-excluding effect of potassium is completely reversed by DOC, which has been shown to inhibit rubidium (or potassium) *binding* rather than *entry*.[5] Thus, it is not unreasonable to assume that sodium exclusion is governed by cytoplasmic features which are also concerned with the *binding* of potassium. Similar conclusions have been more or less explicitly expressed elsewhere by proposals for the existence of cytoplasmic structures, with special spatial arrangments and charge distributions, which could selectively incorporate potassium and exclude sodium.[8, 15–17]

The effects of DOC on the potassium-sodium relationships in *Neurospora* are of particular interest in view of the effects of DOC on salt balance in higher organisms. The role of DOC in the mammal is predominantly observed in its control of sodium retention or, more specifically, the reabsorption of sodium from the glomerular filtrate of the kidney. DOC increases the reabsorption of sodium and decreases the reabsorption of potassium by renal tubular epithelial cells; an insufficiency of DOC leads to a loss of sodium and an increase of potassium in the plasma and extracellular fluids. Thus, DOC appears to exert its effect by controlling the passage of sodium through the renal tubular cells. The relationship of DOC, sodium, and potassium in the mammal is strikingly similar to that reported here in *Neurospora*, where it can be considered that the passage of sodium through the mycelium is facilitated by DOC; in the absence of DOC the accumulation of potassium results in a restriction of the passage of sodium through the *Neurospora* cell. Thus, the passage of sodium through the Neurospora cell appears to be dependent on the state of the potassium *binding* system which can be modified by DOC. In view of the similarity in response to DOC with respect to sodium movement in the renal tubular cell and *Neurospora*, and despite the phylogenetic disparity, the speculation can be advanced that the mechanism of DOC action is similar in both cells.

Summary.—The effects of 11-deoxycorticosterone, azide, and exogenous potassium on sodium uptake and intracellular potassium levels in *Neurospora crassa* have been examined. Germinated conidia suspended in buffered saline solutions achieve an intracellular concentration of sodium approximating that of the suspending medium. The uptake of sodium is not accompanied by a corresponding de-

crease in intracellular potassium, and at almost all concentrations of sodium employed no marked change in intracellular potassium was observed. DOC appeared to augment the rate of sodium influx somewhat, but had no pronounced effect on the final level of equilibrium; the presence of DOC caused a modest efflux of potassium. Azide inhibited influx or efflux of sodium, but had no appreciable effect on the levels of sodium already present in the cells; azide also had little effect on intracellular levels of potassium. When potassium was added to the cell suspension it was taken up and accumulated against an apparent concentration gradient. In the presence of exogenous potassium the uptake of sodium was inhibited and the intracellular content of sodium was markedly decreased. The exclusion of sodium accompanying the accumulation of potassium was prevented by the presence of DOC. The implications of these observations are discussed with respect to problems of ion accumulation and exclusion, and to the nature of the hormonal action of DOC.

* This work was supported by a grant from the Commonwealth Fund.

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[6] Preliminary studies had shown that during incubation for several hours no net change of phosphate could be detected in the cells or medium, and washing the cells with saline or water did not alter their phosphate content significantly. It was also established that inulin was not utilized by these cells as a carbon source; moreover, it did not appear to readily penetrate into the cell. Consequently, inulin (0.3%) was incorporated in the medium to serve, along with phosphate, as an indicator of extracellular volume in filtered cells. Aliquots of suspension were filtered, as noted above, the total wet weight determined, and the filtrates were assayed for inulin [Schreiner, G. E., *Proc. Soc. Exptl. Biol. Med.*, **74,** 117 (1950)] and phosphate [Lohmann, K., and L. Jendrassik, *Biochem. Z.*, **178,** 419 (1926)]. The cells were taken up in 0.08 *M* NaCl, mixed thoroughly, filtered and dried. The inulin and phosphate contents of this filtrate were determined, and these values were considered to represent the volume of extracellular medium, which subtracted from the total wet weight gave the weight of the cell water plus the cell dry weight. On the basis of such estimations the cell water was calculated to be about 73 per cent and 69 per cent using inulin and phosphate, respectively, as indicators of extracellular water (these values are the average of 36 determinations). It made only a small quantitative and no qualitative difference in the results whether they were based on values of per cent cell water calculated from inulin or phosphate estimations of extracellular water. Consequently, the average of the values obtained with these two estimations has been used. The difference in estimates for extracellular water with phosphate and inulin might be due to a sluggish movement of inulin into or out of the cell wall, or a slight penetration of phosphate into the cell. The values for per cent cell water varied about ±3 per cent from one experiment to another, but within a particular experiment the variation was less, and appeared to be independent of any of the experimental conditions employed.

[7] The values for Na^{23} in the cells were somewhat higher than what would have been expected on the basis of the Na^{22} analyses, which might suggest a lack of equivalence between Na^{22} and Na^{23}, or that a portion of the Na^{23} already taken up by these cells does not exchange with Na^{22}. However, it seems more likely that the high values for Na^{23} might be due to the presence of some other material(s) in the cell digests which contribute to the readings obtained in the flame photometer analyses for Na^{23}.

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*SULFOLIPID FROM VIRULENT TUBERCLE BACILLI**

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It is known that the bacterial cells of pathogenic human and bovine varieties of *M. tuberculosis* fix the dye neutral red in its salt form (red) to their surface in an alkaline aqueous environment, while mutant strains of these organisms which do not manifest this behavior are highly attenuated or avirulent.[1, 2] This is a preliminary report of investigations providing evidence that the material responsible for this fixation of neutral red is a new type of sulfolipid.

Surface cultures of living bacterial cells from a synthetic medium containing ammonia as the sole source of nitrogen, malic acid (0.3%), glucose (2%), glycerol (0.5%), and the usual inorganic salts, were washed with distilled water after 14 or 28 days of growth. They were briefly extracted, moist, with hexane (Skellysolve B) to which was added some decylamine (0.05%). The extracts were evaporated on a steam bath under N_2 to a small volume and shaken with aqueous 1 *N* citric or 0.1 *N* hydrochloric acid in order to remove most of the decylamine. Such crude extracts of the fully pathogenic strains, H37Rv (human) or Vallée (bovine), when shaken with a dilute aqueous solution of neutral red hydrochloride, became brightly colored red. The neutral red dye is "pulled" into the hexane to form a hexane-soluble salt with the acidic substance or substances dissolved in the hexane; and the amount of neutral red so fixed was measured by appropriate spectrophotometric methods.

Analysis of this reaction led to the conclusion that the material responsible for this "fixation" of neutral red was strongly acidic, and it was assumed at first to be a phospholipid. However, as the work progressed, the phosphorus content of the most abundant neutral red fixing fraction of the crude extracts of virulent strains, separated by chromatography on silicic acid-celite and silica gel or silica gel-celite, decreased strikingly. A test for sulfur revealed its presence in significant amounts. Further purification, including acetone precipitations to eliminate more completely any contamination with phospholipid, has yielded a slightly amber-colored, strongly acid-fast lipid, oily above 25°C, containing 1.1 per cent sulfur and less than 0.02 per cent phosphorus, and fixing 85 mcg of neutral red per mg. This is consistent with about one mole of neutral red fixed in salt form per atom of sulfur, and with the acid equivalent weight of the material, about 3,000. This fraction constitutes 0.1 to 0.2 per cent of the dry weight of the bacterial cells of the strain H37Rv.

Infrared spectrophotometric (IR) and nuclear magnetic resonance (NMR) analyses have been made of this and other similar fractions for comparative purposes, with the generous collaboration of Dr. John C. Craig and Dr. Evan Horning of the Laboratory of the Chemistry of Natural Products, National Heart Institute, National Institutes of Health. In Figure 1 are shown the IR and the NMR curves given by this material. These studies have shown the following:

(1) The material contains a peculiarly high proportion of methyl groups suggestive of the methyl-branched-chain fatty acids described by several previous investigators in hydrolyzed preparations of the acetone soluble lipids of tubercle bacilli.[3, 4, 5]

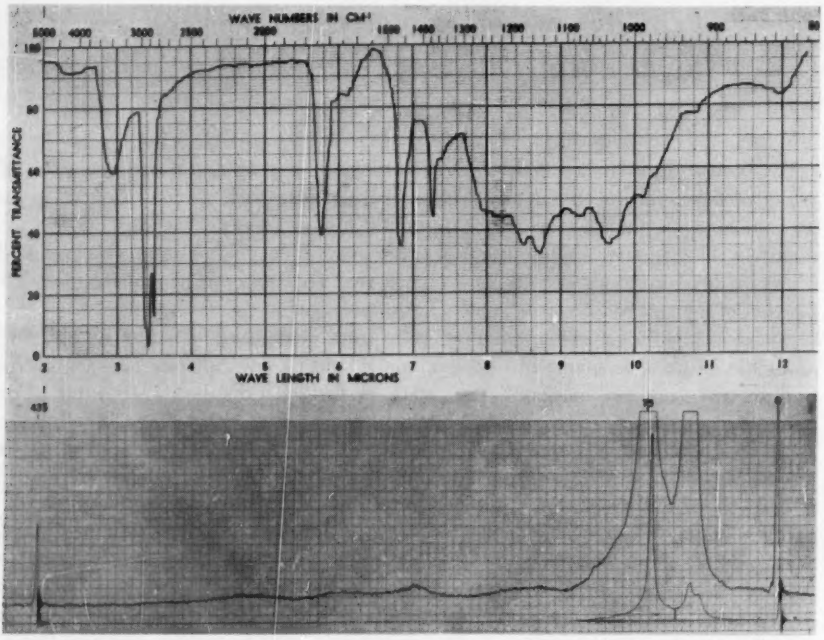

FIG. 1.—(Above) Infrared absorption spectrograph of non-polar sulfolipid fraction from H37Rv, 10% solution in CCl_4, on Model KM-1, Baird-Atomic, Inc., spectrophotometer. (Below) Nuclear magnetic resonance spectrograph of non-polar sulfolipid fraction from H37Rv, in $CDCl_3$, on 60 KC instrument at Varian Associates, Palo Alto, Calif., courtesy of Dr. James N. Shoolery.

(2) It contains a very small proportion of alcoholic hydroxyl groups, which is consistent with its insolubility in water and in methanol, and its high solubility in mineral oil, petroleum ether, chloroform, diethyl ether, and acetone.

(3) The IR absorption bands at 1020–1060 cm^{-1} and 1140–1200 cm^{-1} are consistent with those of a sulfonic acid.

(4) It contains no carbon to carbon unsaturated bonds, which excludes the presence of the phthienoic type of fatty acid.[6, 7]

(5) It contains a prominent carboxylic acid ester absorption band (1,740 cm^{-1}) and little or no free carboxylic acid (1,720 cm^{-1}).

Cells of the H37Rv strain were cultivated on medium containing 1 millicurie of $S^{35}O_4$ per liter. Crude extracts were prepared as described above. They proved

to contain significant amounts of S^{35}, and chromatographic separation of such extracts into various fractions revealed good parallelism between radioactivity and neutral red fixing activity (see Table 1). These results indicate that the prin-

TABLE 1

CHROMATOGRAPHIC ANALYSIS OF CRUDE MATERIAL EXTRACTED BY HEXANE-DECYLAMINE FROM H37Rv ON SILICIC ACID[a]: CELITE (200 MG:100 MG PER MG OF CRUDE MATERIAL), AT 25C; 2 COLUMN VOLUMES OF EACH ELUENT

Eluting Solution	Experiment 1			Experiment 2		
	mg	mcg NR[c]	cpm[d]	mg	mcg NR[c]	cpm[d]
	180	8.2	1,300	84	12.1	1310
Chloroform[b]	120	<0.5	<30	58	<0.5	<30
Chloroform + 4% methanol	32.5	28.5	3,570	12.4	31.0	3,680
Chloroform + 10% methanol	11.6	44.0	3,820	14.5	34.0	3,830
Methanol,[e] 100%	1.4	15.5	715	3.5	38.0	825
Recovery, %	*92*	*97*	*70*	*105*	*99*	*95*

[a] Containing 7.2% H_2O.
[b] Containing 0.75% ethanol.
[c] Expressed as mcg neutral red base fixed per mg of material in hexane.
[d] Expressed as counts per mg per min under standardized conditions, S^{35}.
[e] Most of the neutral red fixing material in this fraction is insoluble in acetone and contains significant amounts of phosphorus.

cipal neutral red fixing material from this strain constitutes either a single molecular species exhibiting much tailing on chromatography or, more likely, a group of closely related sulfolipids with slight differences in polarity. These studies have also revealed that more or less neutral red fixing material of high polarity, soluble in methanol and having low radioactivity, can also be extracted from this and other strains of tubercle bacilli, regardless of their biologic properties or the ability of the whole bacterial cells to fix neutral red in an alkaline aqueous environment. These fractions contained up to 4.2 per cent phosphorus, and their amounts in hexane-decylamine extracts varied in particular with the duration of the extraction period and the age of the cultures, being especially prominent in extracts of old autolyzing populations.

Comparative studies have also been made of other selected strains of *M. tuberculosis* (see Table 2).

TABLE 2

CHARACTERISTICS OF CULTURES AND MATERIAL EXTRACTED FROM FIVE DIFFERENT STRAINS OF TUBERCLE BACILLI

Strains	Cord Formation	Neutral Red Reaction, Whole Cells	Crude Material Extracted with Hexane-decylamine, mg[a]	Neutral Red Fixing Activity per mg of Crude Material, mcg NR[b]	Radioactivity per mg of Crude Material, S^{35}
H37Rv	Strong	Strong	12–17	8–15	1,300 cpm[c]
Vallée	Strong	Strong	12–17	8–15	(Not done)
BCG-P	Strong	Strong	12–17	5–10	120 cpm
BCG-T	Weak	Weak	10–15	0–2	(Not done)
H37Ra	Absent	Absent	10–15	0–2	48 cpm

[a] Hexane-soluble material per gram dry weight of living bacterial cells.
[b] Expressed as mcg of neutral red base fixed per mg of material in hexane.
[c] Expressed as counts per mg per min under standardized conditions.

Extracts have been prepared from two BCG strains of *M. tuberculosis* which are partially attenuated in pathogenicity. One, the least attenuated, fixes neutral red well as whole bacterial cells, and hexane-decylamine extracts of this strain fix at least half as much neutral red as the fully pathogenic strains. However, the sulfur content of the neutral red fixing fractions from this strain is only one-tenth

that of the H37Rv strain; and it appears to produce little or none of the relatively nonpolar neutral red fixing material synthesized by and so easily extracted from the surface of the two fully pathogenic strains. Most of the neutral red fixing activity of the whole bacterial cells and of the extracts of this attenuated strain is fairly attributable to phospholipid.

Cells of the other, more attenuated, strain of BCG fixed neutral red very weakly and yielded crude extract material with one-sixth as much neutral red fixing activity as the less attenuated strain.

In all experiments with the nonpathogenic strain H37Ra, the whole cells of which do not fix neutral red, little or no neutral red fixing material was released into hexane-decylamine extracts, and such an extract contained very little sulfur.

Preliminary tests of the least polar sulfolipid fraction from the strain H37Rv, dissolved in light paraffin oil (Bayol F), and injected by the intracutaneous route into guinea pigs, have shown that it has significant local toxicity both for normal and for tuberculous animals.

Finally, the oily character of this sulfolipid material as well as its apparently superficial location in the bacterial cells would seem eminently to suit the requirements of a capsular or envelope substance responsible for the morphologic serpentine cord formation so characteristic of cultures of neutral red fixing, pathogenic strains of tubercle bacilli.[8] Therefore, it seems reasonable to postulate that this material plays a prominent role in the biology of tuberculosis.

Summary.—Evidence has been presented that the principal material responsible for the fixation of neutral red by fully pathogenic strains of tubercle bacilli consists of sulfolipid. Physical and chemical studies of this material indicate that it is a kind of chemical substance heretofore unrecognized in biological products.

* This work was aided by a grant from the National Institute of Allergy and Infectious Diseases U. S. Public Health Service.

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*ISOLATION OF INFECTIOUS DEOXYRIBONUCLEIC ACID FROM SE POLYOMA-INFECTED TISSUE CULTURES**

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Several examples of ribonucleic acids isolated from purified viruses or virus-infected animal or plant tissue have proved to be infectious.[1–8] On the other hand, findings of infectious deoxyribonucleic acids (DNA) have not been substantiated. Hayes *et al.*[9] have observed leukemias in AKR-C_3H hybrid mice injected with mixed nucleic acids prepared from the leukemic organs of AKR mice. These hybrid mice, however, show a high incidence of spontaneous leukemia.[10] Latarjet *et al.*[11] have reported the induction of multiple tumors in C_3H and AKR mice injected with nucleic acid extracted from the cells of leukemic AKR mice. Elucidation of the nature of the infectious principle in these preparations is yet to be made. (In a personal communication, Dr. R. Latarjet has informed us of his recent results. The preparations appeared to lose their ability to induce tumors in mice after treatment with DNase.) There is strong evidence[12] for the presence of at least two carcinogenic viruses in AKR leukemic mice. Whether the activity of the tissue preparations is due to the presence of infective nucleic acid remains unclear. In view of the difficulties in preparing highly-purified nucleic acids devoid of other cellular or viral constituents, the most convincing evidence for the successful isolation of an infectious nucleic acid rests on its sensitivity to a specific nuclease.

The present report describes the isolation from mouse embryo tissue cultures infected with SE polyoma virus[13] of an infectious nucleic acid resistant to the action of ribonuclease (RNase), but which is inactivated by deoxyribonuclease (DNase). The intact virus is resistant to both enzymes.

The method of culturing the virus has been described.[14] In brief, Swiss mouse embryo cells were grown in 32-ounce bottles, then retrypsinized and grown in 2-ounce prescription bottles. The maintenance fluid consisted of medium 199 and 2 per cent horse serum with the addition of 3.5 ml of 5 per cent sodium bicarbonate solution for each 100 ml of fluid. At the time the virus was inoculated, each bottle contained approximately 3×10^5 cells in a sheet which completely covered one flat surface of the bottle. On harvesting, the cells were frozen and thawed, then pooled, and this constituted the starting material for the preparation of nucleic acids.

The "crude virus suspension" was either an aliquot of pooled cultures used as such or concentrated tenfold by lyophilization. After storage from 1 to 24 hours at 4 to 6°C, aliquots of 50 ml were dialyzed against 3 liters of 0.02 *M* phosphate buffer, pH 7.2, for 24 hours. For the concentrated virus suspensions, a 48-hour period of dialysis, with two changes of buffer, was employed. The infectious titers of the pooled virus varied between 10^{-5} and 10^{-6}, whereas that of the concentrated virus was 10^{-7}.

Two methods of preparing nucleic acids from the tissue cultures were employed:

Method 1: This procedure followed in general that described by Gierer and Schramm.[1] To 50 ml of dialyzed crude virus suspension, an equal volume of 80

per cent phenol, by weight, was added, a fresh bottle being opened for each experiment. (Mallinckrodt, St. Louis, Mo., liquefied phenol, 88 per cent, analytical reagent, was adjusted with 0.02 *M* phosphate buffer, pH 7.2, to obtain the desired concentration.) The virus-phenol mixture was shaken vigorously for 15 minutes at 20°C by machine, and then centrifuged at 3900 *g* at 0°C for 5 minutes. The upper, aqueous, phase was removed and treated with 75 per cent phenol in a similar fashion two further times. The final aqueous layer was freed of phenol by ten extractions with equal volumes of peroxide-free ether at 0°C. The dissolved ether was evaporated in a stream of nitrogen at 0°C.

Method 2: This procedure was an adaptation of that described by Kirby.[15] Solid sodium-*p*-aminosalicylate (6% by weight) was added to the crude virus suspension, which was then shaken with an equal volume of 88 per cent phenol at 20°C for 15 minutes. The extraction was continued by magnetic stirring for 4 hours at 6°C. After centrifugation at 3900 *g* for 5 minutes at 0°C, the remaining procedure followed closely that described for Method 1.

This method was applied to uninfected cultures for the preparation of control nucleic acids.

When tested in tissue cultures, reproducible cytopathogenic effects were obtained in several series of experiments with concentrations varying between 3 and 30 μg of mixed nucleic acid per ml. Intact virus was later identified in these nucleic acid-inoculated cultures by demonstration of cytopathogenic changes and the ability of the supernatant fluids to hemagglutinate[16] as well as to induce tumors in injected animals.[13, 17]

The following conditions appeared to be optimal for the demonstration of nucleic acid infectivity in tissue cultures: (1) removal of the culture medium from the cell sheet followed by two washings with 0.14 *M* NaCl in 0.02 *M* phosphate, pH 7.2. and removal of the washings prior to inoculation of the nucleic acid; (2) adjustment of the nucleic acid solutions to 0.5 or 0.8 *M* NaCl in 0.04 *M* phosphate, pH 7.6; (3) addition of 2.0 ml of nucleic acids (*ca.* 3 to 30 μg/ml as estimated from absorbancy at 260 mμ) to each 2 oz. bottle containing about 3×10^6 cells; (4) incubation of the mixture at 34°C for 20 minutes; and (5) removal of the nucleic acid solutions before adding fresh culture medium. These procedures were fashioned after those used by Alexander *et al.* for the demonstration of infectivity of RNA from polio virus in HeLa cell monolayers.[3]

The results from two typical experiments are presented in the table. Comparable results were obtained with nucleic acids prepared by both procedures. The cytopathogenicity of two specimens before and after treatment with RNase and DNase may be compared with those of the crude virus from which the nucleic acid was prepared. (RNase from Sigma Chemical Co., St. Louis, Mo., 5X crystallized; protease-free. DNase from Worthington Biochemical Corp., Freehold, N.J., 2X crystallized; used in the presence of 0.005 *M* $MgSO_4$.) Uninfected cultures and nucleic acids prepared from them are shown as controls.

Exposure to RNase or DNase (3 to 30 μg/ml final concentration) did not affect the infectivity of the "crude virus," nor was the infectivity of the nucleic acid preparations affected by treatment with RNase for 15 minutes at 20°C. In contrast, exposure of the nucleic acid preparation to equivalent concentrations of DNase for an equal time completely destroyed the infectivity. The last four columns of the

INFECTIVITY OF SE POLYOMA VIRUS NUCLEIC ACID AND SE POLYOMA VIRUS FOR MOUSE EMBRYO CELL CULTURES AND FOR HAMSTERS

Method	Preparation	Days on which mouse embryo cell cultures showed cytopathogenicity[a] — 1st tissue culture passage							Days on which mouse embryo cell cultures showed cytopathogenicity[a] — 2nd tissue culture passage						Hamsters injected with 1st or 2nd tissue culture passage fluid			
		7	14	21	28	35	42	63	7	14	21	28	35	42	No. injected	No. lost	No. with tumors	Period of observation (days)
I	Nucleic acid held at 20°C for 20 min.		2						0	2					11	0	11	18
			±	3	4													
	treated with DNase		0	0	0				0	0	0	0	0	0	11	0	0	67[c]
	treated with RNase (3 μg enzyme/ml at 20°C for 15 min.)		3	3	4				0	0	3	4			12	5	7	28
II	Nucleic acid[b]	1	1	3					3	4					10	3	7	18
	treated with DNase	0	0	0	0	0	0	0	0	0	0	0	0	0	15	0	0	88[c]
	treated with RNase (30 μg enzyme/ml at 20°C for 15 min)	0	0	2					3	3	4				13	7	6	22
	Virus	1	4						0	2	3				7	1	6	41
	treated with DNase	1	4	4					0	3	4				13	0	12	39[c]
	treated with RNase (30 μg enzyme/ml at 20°C for 15 min.)	1	3	4					0	2	3				13	2	11	30
II	Nucleic acid from non-infected mouse embryo cell cultures	0	0	0	0				0	0	0							
	Control noninfected mouse embryo cell cultures	0	0	0	0				0	0	0							

[a] Cytopathogenicity scored: 0 = no degeneration to 4 = complete degeneration.
[b] Nucleic acid obtained from 10-fold concentrated virus infected tissue cultures.
[c] These animals remain under observation.

table contain the results of the inoculation of the fluid from the nucleic acid-infected tissue cultures into hamsters. A high percentage of these have developed tumors. The remainder are still under observation.

Detailed data on hemagglutination and results of the direct inoculation of the nucleic acid preparations into animals will be reported later together with the analytical and physical-chemical properties.

Studies on the distribution of the SE polyoma virus in mouse embryo cells by means of the fluorescent antibody technique[18] have revealed that the nuclei are first affected by the infection. Electron-microscopic examination of such cells reveals an extensive localization of the virus particles within the nuclei.[19] These findings together with the presence of a DNase-sensitive, RNase-resistant, infectious component which can be extracted and purified from a virus suspension suggest that the nucleic acid of the SE polyoma virus is DNA.

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CONTENTS

Page

GEOPHYSICS.—SYMPOSIUM ON PHYSICS, CHEMISTRY, AND BIOLOGY OF THE ATMOSPHERE . 1649

CHAIRMAN'S INTRODUCTORY REMARKS . . . . . *By Horace R. Byers* 1649

HYDRODYANAMICS OF THE ATMOSPHERE AND NUMERICAL WEATHER PREDICTION—A SYNTHESIS . . . . . *By Jule G. Charney* 1650

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BIOCHEMISTRY.—ON BIOCHEMICAL EVOLUTION: LYSINE FORMATION IN HIGHER PLANTS . . . . . *By Henry J. Vogel* 1717

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GENETICS.—CHARACTERISTICS OF A HIGH FREQUENCY OF RECOMBINATION (HFR) STRAIN OF *Salmonella typhosa* COMPATIBLE WITH SALMONELLA, SHIGELLA, AND ESCHERICHIA SPECIES . . . . . *By L. S. Baron, W. F. Carey, and W. M. Spilman* 1752

MATHEMATICS.—ON FINITE GROUPS OF EVEN ORDER WHOSE 2-SYLOW GROUP IS A QUATERNION GROUP . . . . . *By Richard Brauer and Michio Suzuki* 1757

MATHEMATICS.—GENERALIZED SPACES OF GENERAL RELATIVITY . *By Luther P. Eisenhart* 1759

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MICROBIOLOGY.—HEREDITARY DEFECTS IN GALACTOSE METABOLISM IN *Escherichia coli* MUTANTS, II. GALACTOSE-INDUCED SENSITIVITY . . . . . *By Michael B. Yarmolinsky, Herbert Wiesmeyer, Herman M. Kalckar, and Elke Jordan* 1786

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